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ASSESSMENT OF INDIVIDUAL DIFFERENCES IN CARCINOGEN METABOLISM

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MATH W. A. C. HUKKELHOVEN

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. J. H. G. I. GIESBERS VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN IN HET OPENBAAR TE VERDEDIGEN OP DONDERDAG 21 JUNI 1984 DES NAMIDDAGS TE 4.00 UUR

DOOR

MATHIAS WILHELMUS ARNOLDUS CORNELIS HUKKELHOVEN

geboren te Echt

krips repro meppel

PROMOTOR : PROF. DR. H. BLOEMENDAL
CO-REFERENT: DR. A. J. M. VERMORKEN

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Voor Kitty

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Het vakbekwame werk van de afdelingen Medische Fotografie en Illustratie, met name van dhr. Berris heeft geresulteerd in de fraaie illustraties in dit proefschrift. Dhr. de Graaf van de Medische Bibliotheek dank ik voor de hulp bij het literatuuronderzoek.

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PART 1

GENERAL INTRODUCTION AND AIM OF THE INVESTIGATIONS

1

General Introduction and Aim of the Investigations

EXOGENOUS AND GENETIC FACTORS AS CAUSES OF CANCER

The large interest which has developed both among scientists and lay persons in chemical carcinogenesis can be traced back partly to the observations in the nineteensixties that most human tumors are influenced by exogenous factors (32,70). All conceivable exogenous influences were included in this estimation, not only chemicals and other agents produced by the industry, but also naturally occurring chemicals, radiation and factors associated with lifestyle and personal habits such as cigarette smoking, alcohol use and diet. The statement that perhaps 80-90% of all cancer in the world today is environmentally caused is largely based on worldwide cancer statistics, which show greatly different incidences for various types of cancer in different countries and even within countries (18). That genetic factors are not the primary cause of this geographic variation has been shown by elegant studies of Haenszel et al. (24-27). Their best known - and already almost classical - study involved the examination of incidences of colon and stomach cancer among Japanese living in the United States (24). Colon cancer is a very common neoplasm in the U.S. while stomach cancer is rare. In Japan the situation is reversed: colon cancer is less than half that in the U.S., while stomach cancer is quite common. The investigation revealed that among Japanese whose families had immigrated during the previous generation, the incidence of colon cancer approached that of the U.S. population. Stomach cancer had declined in the mean time, so that by the second generation, the incidence was comparable to the U.S. situation. Since major genetic alterations would require considerably longer than one or two generations, changes in the environ-

ment - probably adoption of the western dietary habits - must be responsible for the increase in colon cancer and the decrease in stomach cancer. Studies such as those for the Japanese migrants to the U.S. suggest that environmental factors are probably more important than heridity in the etiology of colon and stomach cancer. Although for most cancers the environmentally causative agents are not known, some cause and effect relationships are very well documented. The most striking examples include the relationship between cigarette smoking and lung cancer (64,65), the high incidence of leukemia among atomic bomb survivors (10), the occurrence of thyroid tumors among people who were irradiated in the throat region in their childhood (31), the mesothelioma tumor in asbestos workers and angiosarcoma in workers exposed to vinyl chloride.

Although strong evidence points to environmental factors as major causes of human cancer, genetic factors may also play a role in the development of the disease. There are a few forms of cancer which are inherited by pure Mendelian genetics. Such cancers are very rare and include Gardner's syndrome, a type of colon cancer which is inherited in an autosomal-dominant way (20). Another rare genetic disease is xeroderma pigmentosum (12), which inherits in a clear autosomal-recessive mode. Patients with this disorder tend to develop skin cancer upon exposure to the sun, due to an extreme sensitivity to sunlight. In general the incidence of the more general cancers, such as those of lung, breast, colon and uterus is not strongly influenced by genetic factors. This does not implicate that heridity is unimportant in these frequently occurring diseases. Familial components of some of these cancers have been identified in recent years. With breast cancer, for example, women who develop tumors in both breasts and at younger ages appear to have more often relatives who also have breast cancer (1). For colon cancer a subgroup of patients has been described which exhibits a lower age of onset of the disease, more than one primary tumor and a positive family history of the disease (2). For lung cancer it has been found that the incidence is 2- to 3-fold higher in relatives of lung cancer patients than in relatives of control persons (62,63). Smokers from high risk families showed 14-fold higher relative risks than non-smoking persons from control

families. Another line of evidence for familial components in cancer stems from the existence of families in which certain combinations of tumors are found in nearly half of the family members. Combinations that have been described include: ovary and breast tumors (43), breast and gastro-intestinal tumors (44), colon and endometrium tumors (45) and a combination of breast, leukemia, sarcoma, brain, laryngeal and adrenal cortical tumors (46).

The picture as it results from our current knowledge shows that both genetics and the environment play a crucial role in the origin of cancer. The dose-dependent relationship between cigarette smoke and lung cancer is well established, yet not everyone who smokes develops bronchial carcinoma. In which part of the multi-step process leading to cancer, these genetic factors are expressed is not known. At least five steps can be distinguished in this process:

1 The causative agent (usually a carcinogenic chemical) enters the body, mostly through surface epithelia.

2 It is converted enzymatically both to inactive products which can be eliminated, and to active carcinogenic forms.

3 The latter can combine with cellular macromolecules and may - in the case of DNA - cause heritable damage to the cell. This damage can possibly lead to escape of the commitment of cells to senescence (i.e. limited lifespan) leading to a capacity for infinite multiplication preceding malignant transformation.

4 The transformed cell divides to form a tumor.

5 The tumor can subsequently metastasize throughout the body.

The exact outcome of each of these steps depends on a large number of factors which are determined by the genetic constitution of the person exposed to the chemical. The balance of the activating vs. detoxifying enzymes affects the relative amount of ultimate carcinogen formed (see also page 16). The organism also has the ability to repair some of the damage caused by the interaction of the carcinogen with the target molecules in the cell. The transformed cells may display surface antigens which are recognized by the host immune system leading to their destruction. Thus the ability of an individual to synthesize enzymes which metabolize carcinogens and others which repair the damage caused by carcinogens could affect cancer susceptibility, as could the proper function-

ing of the immune system. In recent years much progress has been made in understanding carcinogen metabolism and the enzymes which mediate these reactions. Of one of these enzymes, aryl hydrocarbon hydroxylase - possible a key enzyme in chemical carcinogenesis - the genetic control has been studied in inbred strains of mice and hybrids. Both aspects - carcinoger metabolism and its genetic regulation - will be discussed in the following section. Attention will be focused to the polycyclic aromatic hydrocarbons (PAH), a uniquely important class of carcinogens because of their prevalence in the environment and their exceptional tumorigenic potency.

CARCINOGEN METABOLISM

Early studies have already indicated the higher incidence of specific cancers in individuals with particular chemical exposures. Well known examples include the high incidence of cancer of the skin of the scrotum among chimney sweeps (54) and bladder cancer in workers exposed to so-called 'aniline' dye (57). These observations stimulated efforts to induce tumors in experimental animals by application of the implicated chemicals or related substances. Yamagiwa and Ichikawa were the first who succeeded in inducing tumors on the ears of rabbits by repeated applications of coal tar (23). The induction of cancer by tars and extracts of tars led to searches for the active agents. In 1933 Cook, Hewett and Heiger (16) identified the first carcinogenic component of coal tar, benzo(a)pyrene (BP). From then on a large number of carcinogenic PAH have been identified. Benzo(a)pyrene, 3-methylcholanthrene, dibenz(a,h)anthracene and 7,12-dimethylbenz(a)anthracene are among the most widely studied PAH in this early period. The molecular structures of some important PAH are shown in Fig. 1.

Efforts have been made to relate molecular structure of PAH to biological activity. Although success was only moderate, certain correlations did emerge. Molecules with the highest biological activity have 4-6 fused aromatic rings and an unsubstituted C-C bond flanked by two aromatic rings. This bond, known as the K-region, is characterized by its relatively high electron density

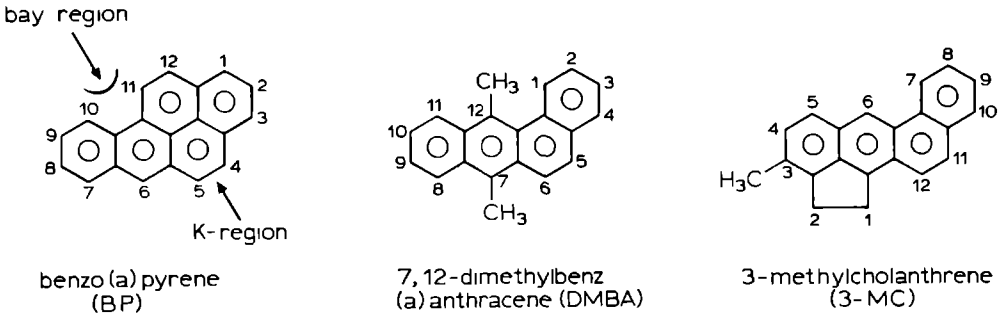

Fig. 1 Molecular structures of some important polycyclic aromatic hydrocarbons

and olefin-like character. Extensive quantum mechanical theories tried to correlate calculated indices of electron density in various regions of the PAH-molecule to bioactivity. In general these theories have been unsuccessful and the reason is - as we now know - that they all failed to take into account the important concept of *metabolic activation*.

It is now generally accepted that practically all xenobiotics are not active in their parent nonmetabolized form but have to be metabolized before they can exert their toxic, carcinogenic, mutagenic or pharmacological actions. This process is called toxification. Most xenobiotics are fat-soluble and they would remain in the body indefinitely if they were not metabolized to more polar derivatives. The enzyme systems which are responsible for this process are principally present in the liver, but they probably also occur in virtually all tissues of the body.

The first step in the metabolism of PAH occurs on the microsomes of the endoplasmic reticulum and is catalyzed by the mixed function oxidase (MFO) enzymes. This system involves at least two protein components:

1 cytochrome P-450 (P-450). This protein represents a family of hemoproteins possessing catalytic activity towards many different substrates, among which drugs, pesticides, steroids, fatty acids, various chemical carcinogens etc. It is believed that several forms of P-450 exist but the estimations range from 3 to about 20 different subspecies of P-450 (58). This means that overlapping substrate specificity accounts for all diversity observed in the metabolism of thousands of different

chemicals.

2 A flavoprotein called NADPH-cytochrome P-450 reductase (sometimes also designated as NADPH-cytochrome c reductase).

Both the P-450 and the reductase enzyme require NADPH (or NADH) as cofactor and molecular oxygen as substrate.

The result of MFO action on PAH is the insertion of one atom of atmospheric oxygen into the molecule. After this first activation step many other pathways for the arene oxide are possible. It may rearrange nonenzymatically to phenols, undergo enzymatic hydration (catalyzed by epoxide hydrolase) to dihydrodiols or become conjugated with glutathione by the enzyme glutathione-transferase. The phenols can be enzymatically coupled to glucuronic acid or sulfate and excreted as the water-soluble glucuronic and sulfate esters. The glutathione conjugates are further metabolized leading to loss of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of the cysteinyl residue. The resulting mercapturic acid is then also excreted. Finally, further oxidative metabolism of the phenols will generate quinones.

Because the urinary metabolites are generally inactive, it was assumed until about 1964 that the metabolism of PAH was exclusively a process of deactivation and detoxification of potentially harmful compounds. From then on evidence accumulated which pointed to a role of metabolism in mutagenicity and/or carcinogenicity of PAH. A number of findings were essential for this hypothesis and for the identification of the ultimate carcinogenic metabolites:

1 When [^{3}H]labeled carcinogenic PAH were applied to mice *in vivo* or incubated with mouse embryo cells *in vitro*, a small fraction of the compound became covalently bound to cellular macromolecules. The extent of binding to DNA and RNA, but not to proteins correlated with the carcinogenic potency of the PAH (11,19). Furthermore it was established that the binding to DNA required the metabolic action of MFO. From these and related experiments it was concluded that cellular metabolism and subsequent binding to DNA was responsible for the carcinogenic action of PAH. Moreover, it was predicted at that time that simple arene oxides were the species that actually bound to DNA.

2 When BP-oxide was allowed to react with DNA in aqueous solution and the resulting products enzymatically degraded to individual deoxyribonucleosides they were not identical to the deoxyribonucleosides obtained when BP was incubated with living cultured cells as analyzed by HPLC (6). With other arene oxides the same lack of correspondence was observed (8). It was concluded that simple arene oxides where *not* the metabolites of carcinogenic

PAH which bind covalently to DNA in living cells.

3 In 1973 a candidate for the role of ultimate carcinogen of BP was predicted from the observation that the 7,8-dihydrodiol metabolite of BP was metabolized by microsomes to a reactive intermediate which bound to DNA (9). It was suggested that this metabolite was a diol-epoxide formed by enzymatic oxygenation by MFO of the 9,10-olefinic bond of 7,8-dihydrodiolBP, leading to 7,8-dihydroxy-9,10-epoxyBP (BPDE).

4 The reaction of anti-BPDE with isolated DNA in a test tube resulted in the same major BP-DNA adduct as reaction of BP with living cells (33).

These four types of experiments led to the hypothesis that PAH exert their mutagenic or carcinogenic action by cellular metabolism of the parent compound and that the critical step in PAH-carcinogenesis resulted from the covalent binding of BPDE to DNA.

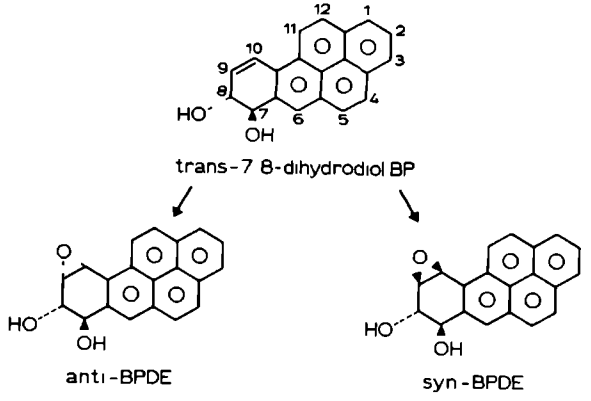

Fig. 2 The anti- and syn-isomer of 7,8-dihydrodiol-9,10-epoxyBP (BPDE). These isomers are also often referred to as BPDE I and BPDE II respectively.

Dihydrodiols of PAH are all in trans-configuration and because of specific enzyme action they are also optically pure. In the case of BP all the dihydrodiols are the (-)enantiomers (71,72). By further enzymic action of the MFO, two isomeric diol-epoxides from (-)-trans-7,8-dihydrodiol are formed, the relative amounts of which depend on the stereoselectivity of the second oxygenation step (73). In the anti-isomer the epoxide oxygen atom and the OH-group adjacent to the aromatic ring are on opposite faces of the molecule, whereas in the syn-isomer these groups are on the same

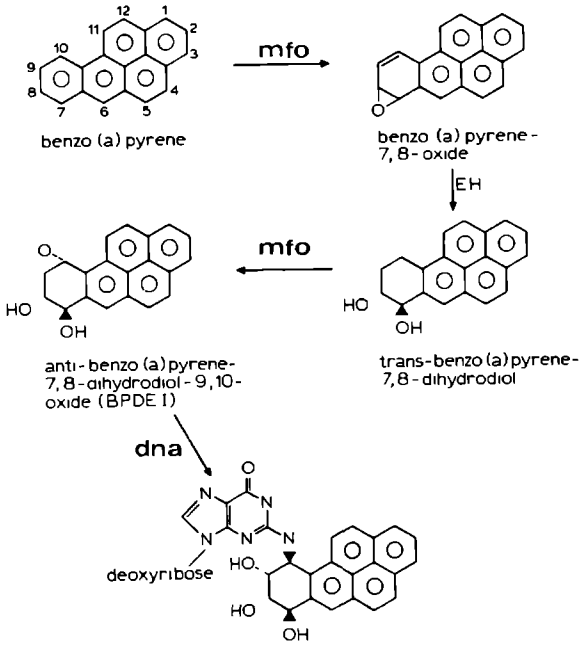

Fig. 3 The metabolic route of BP leading to the formation of the suspected ultimate carcinogen, BPDE I, and the subsequent binding to the exocyclic $-NH_2$ group of deoxyguanosine.

face (Fig. 2). Both anti- and syn-BPDE can exist as optical isomers. Synthesis of the two isomeric syn- and anti-BPDE and separation of the pure (+) and (-) optical isomers of both anti- and syn-BPDE has been achieved (7,29). The principal metabolite formed by living cells appeared to be the anti-isomer. For BP the metabolic route leading to the ultimate carcinogen together with the subsequent binding to DNA is represented in Fig. 3.

The strongest evidence for BPDE as the candidate for the ultimate carcinogen of BP came from comparison of biological activities of different metabolites or derivatives of BP (for a review see 22 and 52). In Table 1 the biological reactivity of various actual and potential metabolites of BP is summarized. From mutagenicity studies it can be concluded that - besides the extremely reactive 7,8-dihydrodiol-9,10-epoxy derivatives - several phenols, the 7,8-dihydrodiol and the 4,5- and 7,8-oxides are active. From these

Table 1 Biological activity of various BP-metabolites*

Metabolite class	Compound	Mutagenicity in: bacteria	animal cells	Malignant transformation	Carcinogenicity
primary epoxides	4,5-	++	+	±	±
	7,8-	+			+
	9,10	+			-
	11,12	+			±
phenols	1	++	±		-
	2	++			++
	3	+	±		-
	4	±			-
	5	-			-
	6	+	±	-	-
	7	±	-		-
	8	-	-		-
	9	±	-		-
	10	-			-
	11	-			+
	12	+			-
trans-dihydro-diols	4,5-	-	-	-	-
	7,8-	+	++	+	++
	7,8- (+)isomer		+		+
	7,8- (-)isomer		++		+++
	9,10-	±	-	-	-
	11,12-	-			-
quinones	1,6-	-	-		-
	3,6 -	-	-		-
	7,8-	-			
	6,12-	-	-		-
diol-epoxides	syn-7,8-diol-9,10-O	+++	+	+	-
	idem (-)isomer	++	+		+
	idem (+)isomer	+	+		±
	anti-7,8-diol-9,10-O	++	++	++	+++
	idem (-)isomer	+	+		±
	idem (+)isomer	+	+++		+++
	syn-9,10-diol-7,8-O	+	-	-	
	anti-9,10-diol-7,8-O	+	-	-	
others	7,8-catechol				-
	7,8-dihydro	++		+	++
	9,10-dihydro	+		-	-
	6-methyl		±		+
	6-hydroxymethyl		±		+
	tetraols		-		-
	triols		-		

Adapted from ref. 52. -O represents an epoxide derivative.

derivatives the 7,8-dihydrodiol and 7,8-oxides appear to have the greatest activity in malignant transformation and carcinogenicity test systems. All data together provide very strong evidence that the 7,8-dihydrodiol-epoxy benzo(a)pyrenes are the most mutagenic, cell-transforming and carcinogenic metabolites. The activity of 7,8-epoxyBP and 7,8-dihydrodiolBP is completely in accordance with this conclusion since they are precursors of the 7,8-dihydrodiol-epoxideBP derivatives. The two other metabolites which do have mutagenic activity - the 4,5-epoxyBP and the 2-phenolBP - are not likely to have much activity in the intact animal: the 4,5-oxide is rapidly hydrated to the inert 4,5-dihydrodiol by epoxide hydrolase (49) while the formation of 2-phenolBP has never been convincingly demonstrated (17).

After the finding that BPDE I is the ultimate carcinogen of BP, research was started to investigate whether analogous diol-epoxide metabolites are also the most active derivatives of other PAH. Much evidence now points to diol-epoxides as the most carcinogenic form of nearly all PAH studied, among which DMBA and 3-MC (29,30, 60). The identification of the ultimate carcinogens of various PAH led to the formulation of the 'bay-region' hypothesis. This theory states that the PAH metabolites with the highest biological activity are diol-epoxides with the epoxide on a saturated angular benzo-ring and forming part of the bay-region of the polycyclic hydrocarbon. From the electronic properties of the diol-epoxides it can be calculated that the epoxides on a saturated benzo-ring undergo ring opening much more easily than do non-bay-region epoxides. The resulting positive charge of the carbonium-ion intermediate is stabilized by delocalisation over the aromatic π-bonds of the adjacent aromatic ring system. The validity of the bay-region hypothesis is also demonstrated by the fact that the more carcinogenic PAH tend to form carbonium-ions more readily from their bay-region diol-epoxides than weaker carcinogens (34,55).

The major DNA-adduct of BP results from covalent interaction at the C-10 position of BPDE I with the exocyclic amino group of deoxyguanosine. The quantitative formation of this adduct has been shown to correlate with mutagenesis in mammalian cells (48) and carcinogenesis in mouse skin (14). A good correlation has also been found between the carcinogenicity of various PAH on

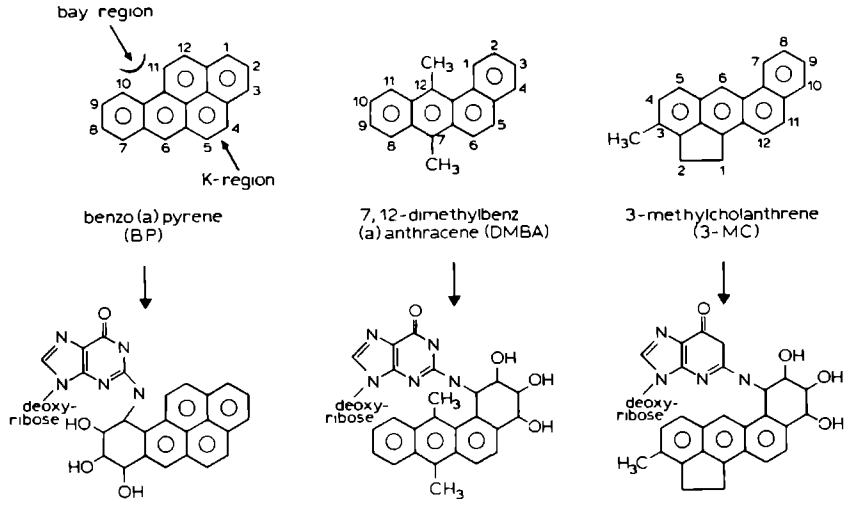

Fig. 4 Structures of the major DNA-adduct of benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene and 3-methylcholanthrene.

mouse skin and the extent of the formation of the respective DNA-adducts in that tissue (53). The structures of the quantitatively most important DNA-bay region diol-epoxide adducts of BP, 7,12-DMBA and 3-MC are shown in Fig. 4. The complete metabolism of BP, as far as it is known at the current state of knowledge, with all possible activation and deactivation steps, is depicted in Fig.5.

GENETIC REGULATION OF CARCINOGEN METABOLISM

We have already discussed the evidence that exists for a role of heriditary factors in the etiology of cancer. It has also been shown that metabolism plays an essential role in the final biological activity of PAH. Therefore it is tempting to speculate that differences in carcinogen metabolism can predispose for cancer. It is well known that the clinical response to drug administration varies widely among individuals (59). For example there are fast and slow acetylators of the widely used antituberculous isoniazid. As a consequence of these interindividual differences the same

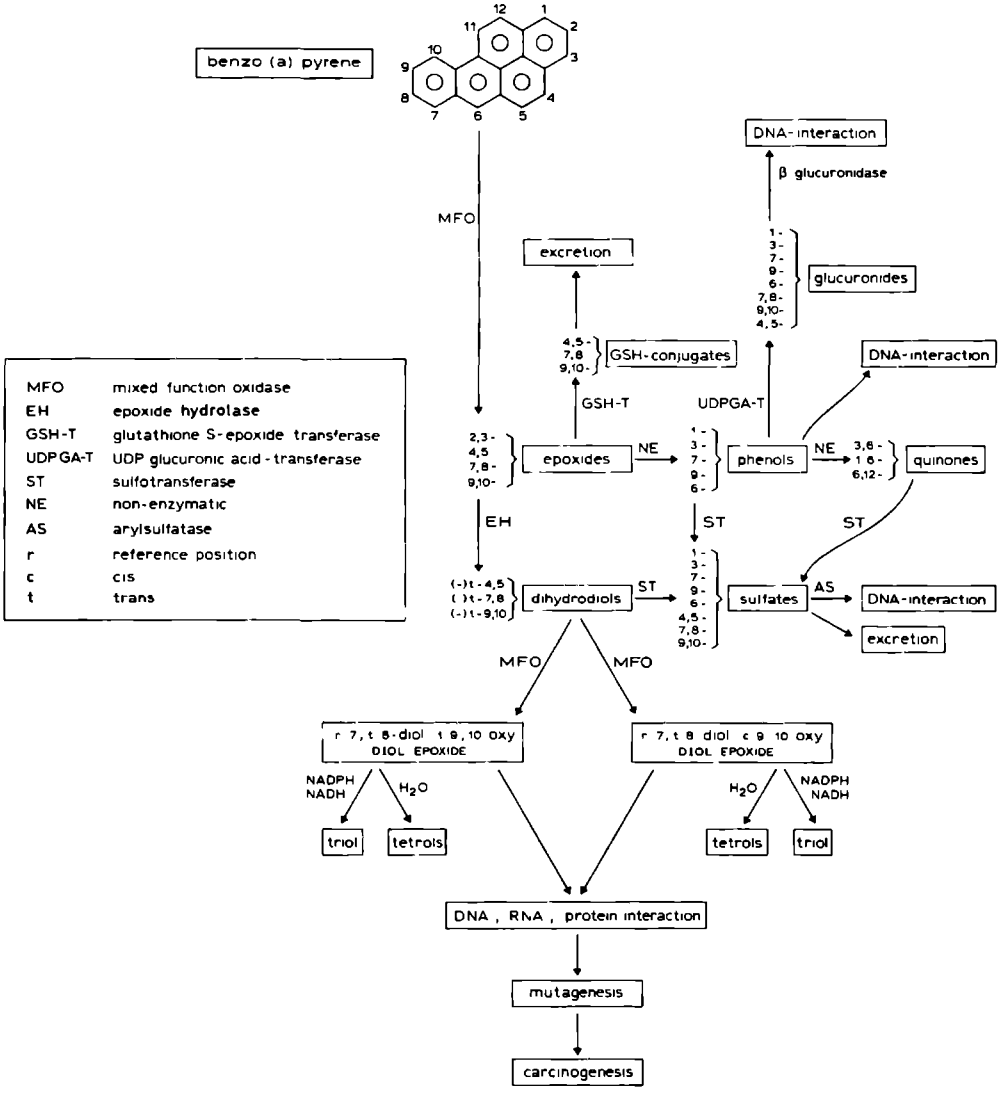

Fig. 5 Metabolism of benzo(a)pyrene as it is known from research on hepatic systems. Adapted from reference 22.

dose of a drug with small therapeutic ratio can be inactive in some patients but may be highly effective or even toxic in others. The implication of this phenomenon is that in clinical practice the achievement of an optimal response to drug administration requires that dosage is closely matched to the patient's individual needs.

The relationship between carcinogen metabolizing enzymes and tumor susceptibility is only beginning to be understood. The most extensively studied enzyme in this respect is one of the enzymes of the mixed function oxidase system, aryl hydrocarbon hydroxylase (AHH) which converts PAH to the primary epoxides and recycles dihydrodiols to the ultimate carcinogens, the diol-epoxides. The enzyme is inducible by PAH but also by other xenobiotics in a large number of mammalian tissues and in a number of mammalian cell-types grown in culture (21,47). Evidence for a role of AHH in susceptibility to tumorigenesis comes from animal experiments, using different inbred strains of mice with genetically determined AHH-inducibility. Although the overall genetic control of AHH-inducibility is quite complex, in some strains the genetics is rather straightforward. With the aid of these strains the importance of carcinogen metabolism has been investigated in detail. Fibrosarcomas initiated by subcutaneously administered 3-MC were shown to be associated with inducible AHH-activity among 14 inbred strains of mice (Fig. 6, ref.61). In another experiment the lungs of two inbred strains of mice (C57 BL/6 and DBA/2) and hybrids of these were exposed to 3-MC by intratracheal installation. Lung lesions occurred more frequently in the inducible strain (C57 BL/6) and in the, also inducible, F_1-generation, compared to the non-inducible strain (DBA/2). The back-cross of F_1-mice to the inducible parents yielded animals with high AHH-inducibility and a high percentage of tumors. The back-cross to the non-inducible DBA/2 parents resulted in both inducible and non-inducible animals, the inducible mice having three times the incidence of lung lesions compared to the non-inducible animals from this cross (39,40). These and other related experiments strongly suggest that the increased susceptibility to 3-MC and BP-induced fibrosarcomas and lung lesions in certain strains of mice is determined by the high AHH-inducibility in these animals.

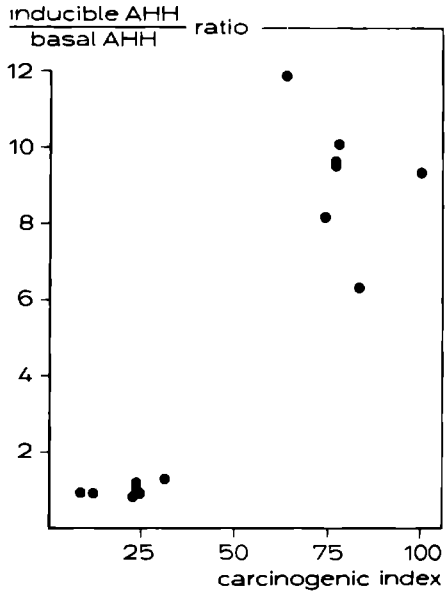

Fig. 6 Relationship between susceptibility to fibrosarcomas initiated by 3-methylcholanthrene (expressed as carcinogenic index) and the genetically determined inducibility of AHH for 14 inbred strains of mice (from ref. 61).

Extrapolation of this finding to the human situation would suggest that AHH-inducibility could also be an important determinant of susceptibility to PAH-induced tumors in man. Investigations in this area became possible when Whitlock et al. (69) described a technique for measuring AHH-inducibility in the easily obtainable blood lymphocytes. The method is based on stimulation of the cells to transform them to lymphoblasts which are rich in endoplasmic reticulum. Then the AHH-activity in the cells is induced with benz(a)anthracene (BA) or 3-MC and the AHH-level compared to that in untreated controls. Using this technique Kellermann et al.(37) reported a trimodal distribution of AHH-inducibility, signifying a single locus of genetic control. In a subsequent study they measured AHH-inducibility in a group of 50 lung cancer patients together with 85 healthy controls and 46 patients with other types of cancer (38). This study revealed

that patients with bronchogenic carcinoma predominantly fell in the classes with higher AHH-inducibility while the majority of the healthy and cancer controls were low to moderately inducible. Hypothesizing that the higher inducibility in cultured peripheral lymphocytes from lung cancer patients reflected a higher level of AHH upon exposure in the target cells of the tracheo-bronchial epithelium, it was concluded that persons with higher AHH-levels (or inducibilities) were at greater risk of developing lung cancer when exposed to PAH (e.g. in cigarette smoke). Since these remarkable results were published a rather large number of studies has appeared which, altogether, have resulted in disagreement about the role of AHH in chemical carcinogenesis.

The genetic control of AHH-inducibility has been confirmed by two twin-studies (5,51). However, the trimodal distribution reported by Kellermann could not be reproduced, indicating polygenic control or too large variability of the lymphocyte test system. Paigen et al. (50) found a low AHH-level in about 50% of the lung cancer patients and no difference in inducibility between healthy controls and the progeny of lung cancer patients. Other reports suggesting a lack of correlation between high AHH-levels in human tissues and susceptibility to lung or laryngeal cancer have also been published (15,35,36,67). Arnott and co-workers tested 800 healthy donors and 600 cancer patients, including approximately 250 lung cancer patients (3,4,56). Although the first results did lend some support to Kellermann's original findings, these data were not convincing. However, when it was realized that the rather large day to day variability in the lymphocyte test system could mask real differences in AHH-inducibility, a different experimental set-up was designed. Blood was obtained from healthy age-matched subjects on the same day that samples were drawn from each lung cancer patient. The results of these case-control studies gave a clear difference in AHH-inducibility between lung cancer patients and controls. When at least three healthy donors were included each day, the patients'inducibility (17.1) appeared to be much higher than that for the controls (7.0). Very recently Kouri et al. (41) carried out a study using an even more rigid experimentation scheme. Many of the factors known to influence the mitogen activation step of lymphocytes were controlled. In a group of 21

lung cancer patients and 30 patients with non-malignant pulmonary diseases, the 14 highest BA-induced AHH-levels were all found in patients with primary lung cancer. Thus, although the relationship between AHH and lung cancer is still unresolved, the most carefully designed experiments strongly point to higher AHH-inducibility ratios or BA-induced AHH-levels in patients with bronchial or laryngeal cancer.

However, it has to be kept in mind that in every study in which a relationship between high AHH-levels and cancer susceptibility has been reported, the AHH-assay was performed on tissues or cells taken from the cancer patients themselves. Therefore, the question that is not answered by these experiments is whether high AHH-levels reflect an inherent susceptibility of an individual to lung cancer or whether lung cancer somehow causes the higher levels of lymphocyte AHH-inducibility. The answer to this question awaits a long-term prospective study in which populations of individuals at high risk are assayed prior to the development of the disease and reassayed after the clinical manifestation of cancer. However, the combined evidence from the described animal experiments and from studies in which the myriad technical difficulties involved in achieving reproducible mitogen activation and subsequent AHH-induction in human peripheral blood lymphocytes are more or less resolved, comprise a stimulation for further investigations concerning genetic variability in carcinogen metabolism and susceptibility to cancer.

AIM OF THE INVESTIGATIONS

In the introductory part of this thesis it has been argued that the major determinants of cancer risk are environmental carcinogens and predisposing host factors. Of the latter, the evidence for a possible role of the genetically determined activity of carcinogen metabolizing enzymes, mainly AHH, has been discussed. Three major draw-backs are inherent in the type of research employed in the elucidation of factors responsible for individual variation in susceptibility to carcinogens.

1 Emphasis has been laid on the role of AHH. However, it has to be realized that by the biochemical assay for AHH only the mixture of alkali-extractable BP-phenols is measured. This assay therefore represents only one part of the complex metabolic system responsible for both detoxification and activation of carcinogens. The complete metabolism of BP is catalyzed by a series of enzymes, of which MFO is only one. Moreover, of the many forms of MFO, AHH is only a single measure. Besides carcinogen metabolism, other parameters may play a decisive role in the multistep process leading to neoplastic development in the airway epithelium, e.g. DNA-repair, tumor promotion and immune surveillance.

2 The majority of the research on individual susceptibility to chemical carcinogens has been carried out using the easily available peripheral blood lymphocytes. However, lymphocytes do not seem to be a target cell-type for PAH-carcinogenesis. Chemical carcinogens can enter the human body by surface epithelia and the majority of their carcinogenic action is indeed exerted in epithelial tissues. Moreover, quantitative differences in carcinogen metabolism between cell-types within a single species have been described (28,42). Therefore, it is important to study biochemical parameters of carcinogen metabolism in human surface epithelia. However, up till now an easily accessible epithelial biopsy tissue suitable for population studies has not been available.

3 The enormous progress which has characterized toxicological research during the last two decades has involved mainly investigations on hepatic systems. In depth characterization of isolated proteins, e.g. cytochrome P-450, has provided powerful tools for the elucidation of action of drug-metabolizing systems. The principles emerging from this research on liver have been extended to other organs up till five or six years ago without much questioning about the validity of this extrapolation. However, it has been realized rather recently that for the explanation of the 95% of extrahepatic tumors in the western world, more extensive research is needed on the relationship between cancer in a specific extrahepatic organ and the genera-

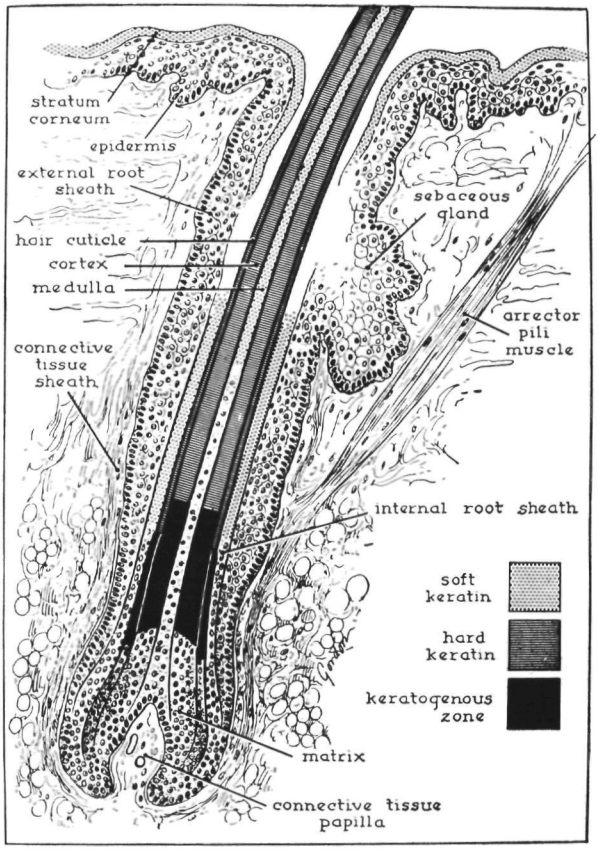

Fig. 7 Diagram of a hair follicle in the skin. (from: A.W. Ham and D.H. Cormack, Histology. Philadelphia: J.B. Lippincott Company, 1979)

tion of potential carcinogens and other relevant processess in that organ.

It has been proposed that human hair follicles might be a convenient biopsy tissue for the evaluation of the role of biochemical parameters of carcinogen metabolism in individual susceptibility to carcinogens (66). These suborgans have already proven to be suitable for detection of some genetic disorders (12). Since they are primarily composed of sleeves of epithelium, continuous with the surface epidermis (Fig. 7), they represent a cell-type highly

relevant for chemical carcinogenesis. Since they are easily available from large numbers of people, they appear to be particularly useful for population studies. Moreover, the recently developed technique for culturing keratinocytes from human hair follicles (68) renders them useful for testing biochemical parameters which require longer incubation times (e.g. induction of carcinogen metabolizing enzymes and isolation of carcinogen-modified DNA). The use of human hair follicles as a model tissue for studies on carcinogen metabolism and for the prediction of an individual's capacity to metabolize carcinogens, represents a new item in toxicological research. Consequently, methods and techniques for measurement of parameters in carcinogen metabolism have not been available up till now. Moreover, knowledge concerning the exact nature of the toxification and detoxification routes in hair follicles or cultured hair follicle keratinocytes has been lacking up till now. The aims of the investigations whose results are presented in this thesis are:

1 To develop methods for the assessment of various biochemical parameters involved in PAH metabolism in hair follicle cells.
2 To characterize PAH metabolism in these cells and to compare it with the target tissue of PAH-induced neoplasia, the tracheobronchial epithelium.

In chapter 2 of this thesis the device of a new culture dish is described. A bovine eye lens capsule serves as the growth substrate for lens epithelial cells. With the aid of this system a routine method for the culturing of hair follicle keratinocytes has been established (68).

Since biochemical parameters, such as enzyme activities, have to be correlated to the amount of biologically active tissue present, a so-called reference variable has to be used. In freshly isolated hair follicles much protein - which is very widely used as a reference variable - is associated with non-living keratinized cells. Therefore we have developed a method that provides the opportunity to measure DNA as a reference variable (chapter 3). DNA is judged to be a good alternative for protein since (1) DNA content depends only on the number of cells present and (2) interference of keratinized cells is unlikely because during the process of keratinization the cell nucleus is eliminated.

In view of the fluorescence properties of many PAH we have realized that a number of enzyme assays that we wanted to develop for hair follicles could possibly be based on measurement of fluorescent end-products. On the other hand the number of cells present in hair follicles is relatively small. Together with the known low specific activity of carcinogen metabolizing enzymes in extrahepatic tissues, the need for equipment to enhance a fluorescent signal was obvious. Therefore, we developed a device in fluorometry which resulted in an increase in the fluorescence detection limit (chapter 4).

Using the fluorescence enhancement technique and the DNA-assay for reference purposes, we developed three assays for enzymes which are thought to play an essential role in PAH-metabolism. First, a procedure for the determination of basal AHH-activities in freshly isolated hair follicles and for BA-induced AHH-activities in cultured hair follicle keratinocytes was developed (chapter 5). However, since epoxide hydrolase also plays a key role in the toxification and detoxification of carcinogens, a method was established for the measurement of this enzyme in hair follicles (chapter 6). As an important representative of enzymes catalyzing conjugative pathways of carcinogen metabolism we chose glutathione transferase using the same substrate as in the assay for epoxide hydrolase, 4,5-dihydro-epoxyBP. We succeeded in developing a very sensitive method of determination of this enzyme (chapter 7). After the establishment of these enzyme assays, each measuring one specific BP-metabolite or metabolite-group, a method was developed for the analysis of the whole spectrum of organic solvent-soluble metabolites of BP, using HPLC-methodology (chapter 8). In the somatic mutational hypothesis, the origin of cancer cells is the consequence of damage or alternations of the genetic material in the cell. According to this theory chemical carcinogens act by an irreversible modification of DNA fragments by metabolites of carcinogens. In chapter 9 a method for the quantification of these DNA-BP adducts in cultured hair follicle keratinocytes is described.

In part 3 of this thesis, evidence for a role of human hair follicles as indicators for individual differences in carcinogen metabolism, is presented. Both the BP-metabolite profiles as analyzed by HPLC and the induction of AHH-activity after BA-exposure

are compared in freshly isolated hair follicles, cultured hair follicle keratinocytes and cells of the human bronchial epithelium (chapter 10). In chapter 11 investigations about genetic factors governing BP-metabolism in hair follicles are presented, and chapter 12 deals with the relation between formation of phenolic and dihydrodiol BP-metabolites in hair follicles.

The aim of the studies described in part 4 was to investigate whether the widely used animal model system for chemical carcinogenesis (C3Hz mice) was comparable with our human model system (hair follicle keratinocytes) and with the human target cells for BP-induced neoplasia (bronchial epithelial cells). For this purpose seven different culture systems have been established: skin fibroblasts, epidermal cells and bronchial epithelial cells from both species and human hair follicle keratinocytes. The response of BP-metabolism towards the important phenol and dihydrodiol metabolites after pre-exposure of the cells to AHH-inducers is described and compared to the response in an *in vivo* experiment using mice (chapter 13 and 14).

Since a number of studies have suggested that induced AHH-activity or the AHH-inducibility ratio might be an important parameter in the determination of individual differences in susceptibility to carcinogens, part 5 of this thesis deals with efforts to measure induced AHH-activity in human hair follicles *in vivo*. First it was investigated whether the administration of potentially AHH-inducing agents when encapsulated in liposomes, can increase their concentration at the application site and decrease their systemic distribution *in vivo*. (chapter 15). It is obvious that notorious carcinogens like chemically pure PAH can not be used for the assessment of AHH-inducibility. Therefore we considered the possible application of low doses of therapeutically used substances to which many dermatological patients are exposed for periods extending many decades, and for which a role as AHH-inducing agent - based on theoretical considerations or literature data - could be assumed. In this context we evaluated the AHH-inducing potential of topically used corticosteroids (chapter 16) and of a commercial coal tar preparation used in the treatment of eczema and psoriasis (chapter 17).

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PART 2

ANALYTICAL PROCEDURES AND TECHNIQUES

2

A Novel Method for Culturing Epithelial Cells on a Biological Substrate

ABSTRACT

A device of a new dish for the culturing of cells on a biological substrate, the eye lens capsule, is described. With the aid of this dish it is possible to investigate the possible interrelationships between the cell substratum and various biochemical characteristics of the cell. It is shown that the protein biosynthetic pattern differs between lens cells cultured on lens capsule as a substrate and cells cultured on foil. Moreover, the new dish offers the possibility to culture epithelial cells on a lens capsule, which provides an alternative to the culturing of these cells on a collagen substrate.

INTRODUCTION

The vertebrate eye lens has been found to be very attractive for studying a wide range of fundamental biochemical processes (2). The organ is composed of long fibers which differentiate from a monolayer of epithelial cells. The whole lens is enclosed by the capsule, a collagen containing structure resembling a basement membrane. This capsule is produced by the epithelial cells which are located beneath the anterior side of the organ. Near the equator of the lens these cells differentiate into the lens fibers.

Successful methods for culturing lens epithelial cells have been described (e.g. 6). In one of the methods, the capsules are placed in a plastic flask. After one week the growing cells reach the edge of the capsules and start to cover the bottom of the flasks (7). It has been demonstrated that the cellular metabolism

differs between cells growing on the capsule and those growing on plastic (8,9). We concluded that the interaction between the cells and their natural substrate, the capsule, is of importance in determining various biochemical characteristics of the cells. In the former experiments it was impossible to have capsules firmly attached during prolonged culturing. Therefore we designed a new culture dish, in which the lens capsule can be stretched. This allows cells to be seeded on the capsule in order to investigate cell-substratum interactions. The new method may also provide an alternative for culturing epithelial cells on collagen (5).

MATERIALS AND METHODS

Chemicals

L-[^{35}S]methionine (specific activity 1180 Ci/mMol) and [^{14}C]methylated protein markers were obtained from the Radiochemical Centre, Amersham, U.K. Fetal calf serum was purchased from Flow Laboratories, Glasgow, U.K.

Tissue preparation

Calf eyes were obtained fresh on ice from the slaughter-house. They were washed with tap water for at least 30 minutes and opened at the lateral side, so that the lenses could be removed without adhering iris material. The lenses were rinsed three times in a calcium- and magnesium-free Tyrode buffer (0.8% NaCl, 0.02% KCl, 0.005% $NaH_2PO_4.H_2O$, 0.1% $NaHCO_3$, 0.2% glucose, adjusted to pH 7.4). Then the lens epithelium was removed. A suspension of lens cells could be obtained by gentle stirring of two capsules per dish in culture medium during approximately one hour.

Culturing of lens cells

After having stretched a lens capsule on the central cylinder of the dish (see Results and Discussion) 0.2 ml of lens epithelial cell suspension, containing approximately 20,000 cells, was added. The culture medium consisted of Eagles Minimal Essential Medium supplemented with 20% fetal calf serum, penicillin (50 I.U. per ml) and streptomycin (50 µg per ml). The cells were cultured at 36.5°C in a 95% air, 5% CO_2 atmosphere. The medium was changed twice a week.

Protein synthesis in cultured lens cells

After cell growth had started the cells were labeled with L-[^{35}S]methionine (10 µCi) in labeling medium (Hank's balanced salt solution supplemented with 10% dialyzed calf serum and amino acids except for methionine). After 16 h labeling the medium was removed and the cells were washed twice with 0.9% NaCl solution. Then the cells were scraped with a bent Pasteur pipette from the capsule or the plastic in 0.9% NaCl solution, and centrifuged at 1000g for 5 min. The cells were solubilized in 1% SDS, 1% 2-mercapto-ethanol and 10 mM sodium phosphate, pH 7.0 and heated at 100°C for 3 min. Analyses were performed by sodium dodecylsulfate polyacrylamide gel electrophoresis according to

Laemmli (4). The slab gel contained 13% acrylamide, 0.4% methylene bisacrylamide and 0.1% sodium dodecylsulfate. Staining and destaining were performed as described by Weber and Osborn (10). For detection of labeled proteins the procedure of Bonner and Laskey (3), was used in combination with the drying method described by Berns and Bloemendal (1).

RESULTS AND DISCUSSION

Lens capsules were isolated as described in the Methods section and stretched in the new developed culture dish constructed from aluminia (except for the central cylinder which is made from stainless steel). Fig.1 shows the way in which the various parts of the dish are put together. The complete technical design with exact dimensions is depicted in Fig.2. Essentially, the bottom of the dish (1 in Fig.1) is covered by a square piece of foil (a in Fig. 1). Then the central cylinder on which the lens capsule (b in Fig.1) is stuck, is placed on the bottom. The cylinder is forced through the opening of the bottom by gently pressing of the upper part of the dish. After placing the lid the various parts are fixed together by means of four screws. Upon inoculation of a lens epithelial cell suspension into the central cylinder, the cells start to grow on either plastic or capsule.

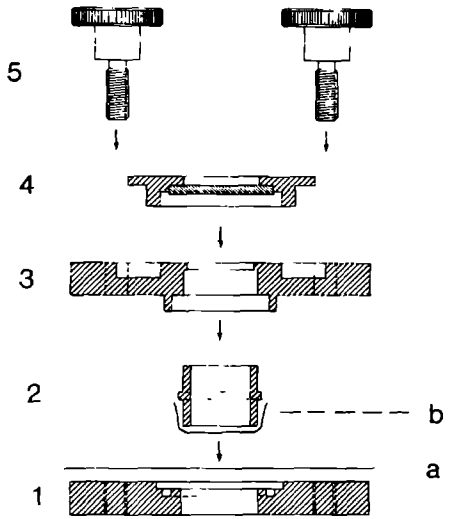

Fig. 1 The various parts of the dish and how they are put together. The explanation is given in the text above.

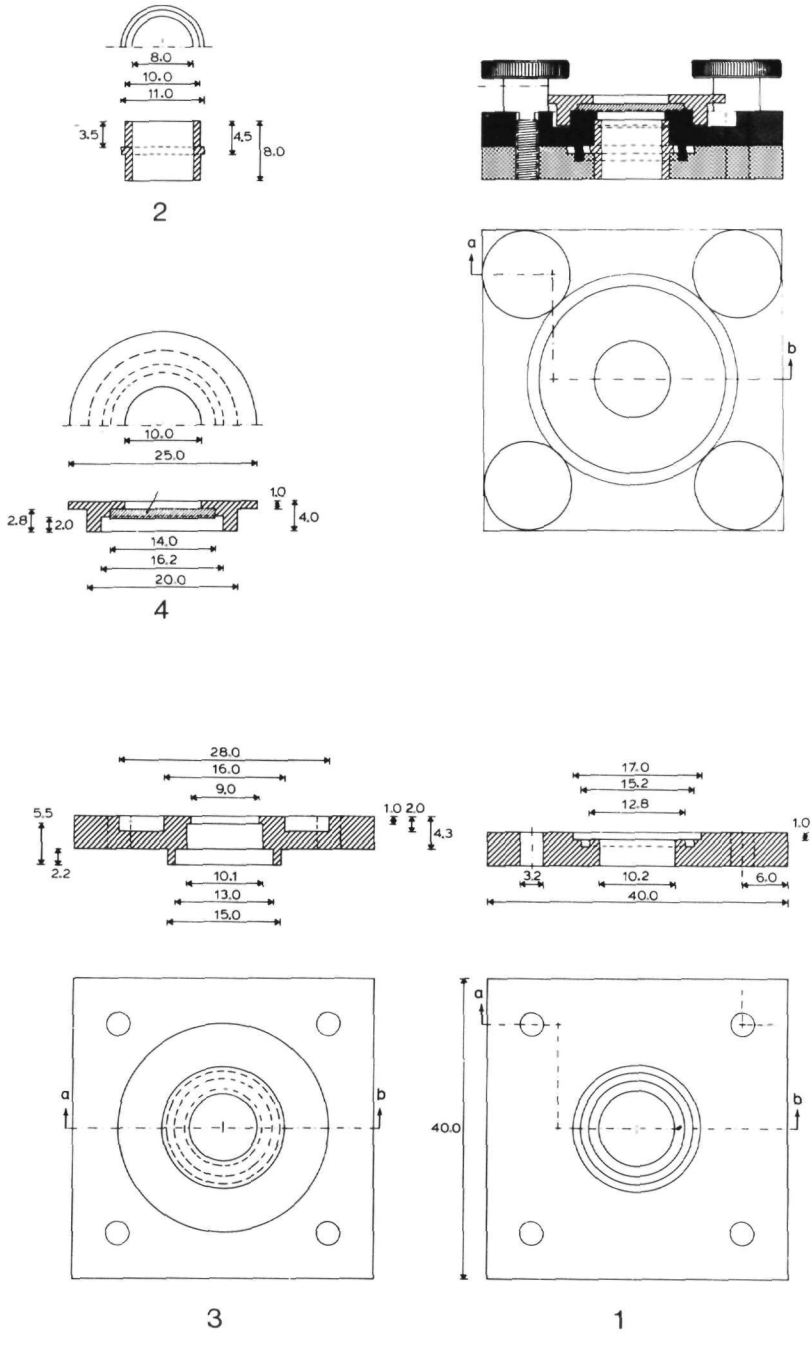

Fig. 2 The technical design of the dish. Bold type numbers correspond to the parts of the dish shown in Fig. 1. Small figures are dimensions in mm.

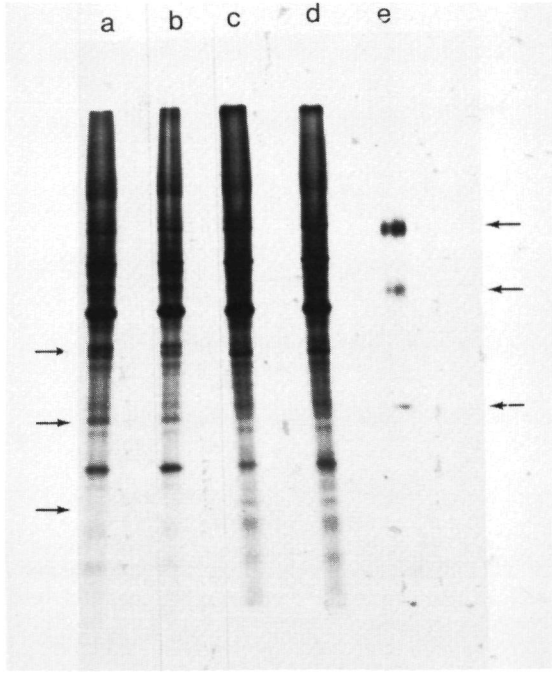

Fig. 3 SDS polyacrylamide gel electrophoresis of [^{35}S]-methionine labeled lens cells cultured on foil (lanes a,b) and capsule (lanes c,d). Lane e contains marker proteins: bovine serum albumin (M=67,000), ovalbumin (M=45,000) and carbonic anhydrase (M=30,000). (right arrows). Left arrows indicate protein differences between cells cultured on capsule and foil.

The sodium dodecylsulfate-gel electrophoretic patterns of [^{35}S]-methionine labeled proteins of cells cultured on capsule and on foil are shown in Fig. 3. Various differences can be seen between the cells grown on capsule and on plastic (for instance in the molucular weigth region of 38,000, 29,000 and 22,000). We have described previously biochemical differences between lens cells growing on capsule and plastic (8). In our new culture system the protein biosynthetic patterns of lens cells grown on plastic and capsule are also different. Thus, the interaction between lens cells and their natural substrate, the lens capsule, is responsible for at least some biochemical features of the cells in culture. The new dish offers the possibility to investigate which

factors in the capsule are responsible for the observed differences. For instance, the capsule can be treated with proteolytic enzymes in order to verify whether this treatment affects the protein biosynthetic characteristics of the cells. In another paper (11) we have shown that the new dish with the fixed lens capsule can offer an alternative for the culture of epithelial cells on a collagencoated substrate, as described by Liu et al. (5) or for the 'feeder cell' technique which depends on the support of a layer of lethally irradiated 3T3 cells (7). The most attractive application of the new dish is the culturing of human keratinocytes from scalp hair follicles without contamination with dermal cells.

Application of the dish concept for commercialization

The large response of investigators to our original article describing the new culture technique for epithelial cells, motivated us to make the dish commercially available. For this purpose Sanbio B.V. at Nistelrode, The Netherlands, was found willing to cover the marketing and sales aspects of this project. Since it was realized that bovine eye lens capsules are not easily available for every investigator, the firm also decided to produce the capsules for commercialization. As a result the complete technique for culturing epithelial cells (e.g. human hair follicle keratinocytes) is now available for each laboratory. Since the original prototype (Epicult I) which has been described in this chapter, some modifications in the model have been made. Together with the larger production lots this has resulted in an acceptable price level. The new dish (designated as Epicult II) has the following alterations compared with the prototype model:

1. The dish has been made round instead of square.
2. The construction of the bottom and upper plate as well as the lid has been more simplified.
3. Only two instead of four screws are necessary to fix the various parts of the Epicult II.

Fig. 4 illustrates the subsequent steps in preparing the Epicult II for use in the initiation of a culture of human keratinocytes originating from hair follicles.

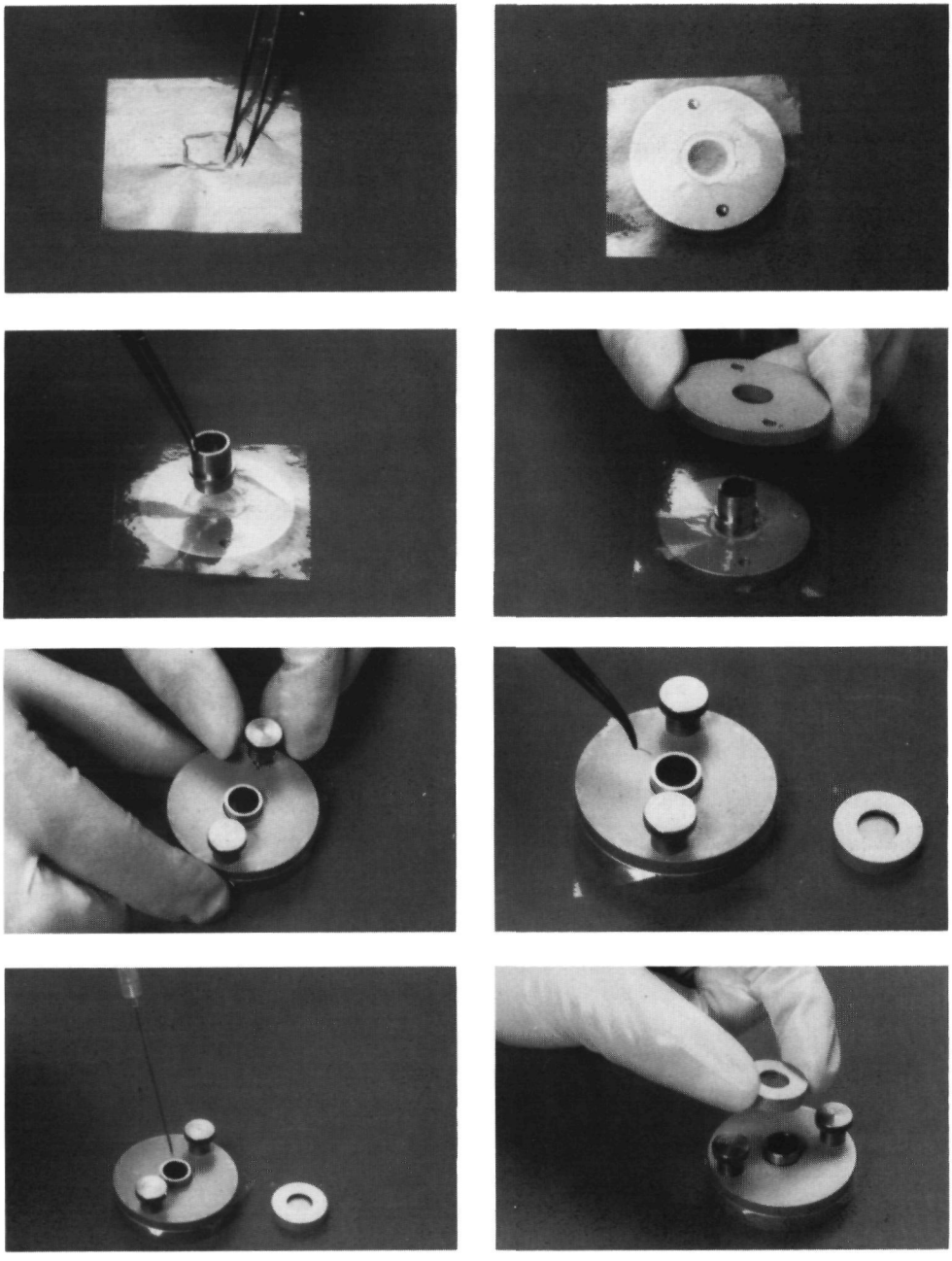

Fig. 4 The procedure for the use of the new version of the Epicult in the initiation of a culture of human hair follicle keratinocytes.

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3

A Simple Fluorometric Microassay for DNA in Hair Follicles or Fractions of Hair follicles

ABSTRACT

Human hair follicles may be useful for determining individual differences in the metabolism of polycyclic aromatic hydrocarbons in epithelial tissues. For quantitative measurements of carcinogen metabolism, determination of the amount of DNA in the hair follicles is necessary in order to correct for individual size variation. A simple method for DNA determination in hair follicles is described, based on the use of a hypotonic pronase solution to solubilise DNA which is then available for complex formation with mithramycin or 4,6-diamidino-2-phenylindole. 2HCl (DAPI). The mithramycin method permits the measurement of DNA in a small number of hair follicles, while the DAPI method is sensitive for as little as one single bulb.

INTRODUCTION

Hair follicles are currently used for the determination of genotypes in inborn errors of metabolism (1,9). Moreover, they can be useful for certain fields of pharmacology and toxicology. We have shown recently that polycyclic aromatic hydrocarbons (PAH) can be metabolized in the isolated human hair follicle (7). After incubation with benzo(a)pyrene, the major organic soluble metabolites formed in the hair follicle are 3-hydroxybenzo(a)pyrene, 7,8-dihydrodiolbenzo(a)pyrene and 9,10-dihydrodiolbenzo(a)pyrene. The formation of the second metabolite is important because this intermediate is believed to be the direct precursor of the ultimate carcinogen of benzo(a)pyrene, the 7,8-dihydroxy-9,10-epoxy-

benzo(a)pyrene (10). As hair follicles are derived from the epithelium, the target tissue of most human malignancies, they constitute a biopsy material particularly useful for studying the mechanisms of the chemical induction of carcinomas. A problem in using hair follicles is that their sizes may vary considerably among individuals and also during the cycle from anagen to telogen hair.

Grimm et al.(2) used single hair roots for heterozygote detection in Fabry's disease, employing protein content as a reference variable. Their method is both time-consuming and inaccurate because of the protein contribution of keratinized cells. Vermorken et al. (6) have used reference enzymes to correct for individual variation in hair follicle size. This method requires search for an enzyme with comparable histochemical distribution in the hair follicle. This is a laborious task which can not always be accomplished. The measurement of DNA in individual hair follicles was thought to be an alternative because DNA content depends only on the number of cells present and interference of keratinized cells is unprobable since during the process of keratinization the cell nucleus is eliminated. In this chapter we describe a rapid method for DNA determination in single hair follicles, which is essentially a modification of the method of Hill and Whatley (3). For the determination of DNA in hair follicle fractions an adaptation of the method of Kapúscińsky and Skoczylas (4) is described.

MATERIALS AND METHODS

Chemicals

Mithramycin (Mythracin®) was purchased from Pfizer Ltd. (New York, USA) and 4,6-diamidino-2-phenylindole. 2HCl (DAPI) was obtained from Serva (Heidelberg, FRG). Pronase, free of nucleases, was purchased from Calbiochem (Lucerne, Switzerland) and herringsperm DNA from Sigma (St. Louis, USA).

DNA assay for a small number of hair follicles

Hair follicles were plucked from random areas of the scalp and those with a visible bulb and sheath were immersed in 950 µl pronase solution (500 µg/ml water). They were incubated in a shaking water-bath at 37°C for 60 min. After the incubation, 50 µl of a mithramycin stock solution (200 µg mithramycin/ml in 300 mM $MgCl_2$) was added. After mixing,the samples were centrifuged and the

fluorescence measured using a Perkin Elmer 3000 spectrofluorometer, at an excitation wavelength of 440 nm and an emission wavelength of 540 nm. Duplicate herringsperm DNA standards were prepared as follows: 1, 2, 3, 4 and 5 µl of a DNA solution (0.5 mg/ml) plus duplo blanks were mixed with 950 µl pronase solution (500 µg/ml water). After incubation, 50 µl of the mithramycin stock solution was added, and the fluorescence determined as before.

DNA assay for fractions of hair follicles

Hairs were plucked and dissected into four fractions: bulb, elongation zone, sheath and shaft. These fractions were immersed in 50 µl of a pronase solution (500 µg/ml water) and incubated in a shaking water-bath for 60 min at 37°C. After the incubation, 1 ml of a DAPI solution in 0.01 M NaCl buffered with 0.005 M Hepes (pH 7.0) was added. The concentration of DAPI was 50 ng/ml, except for the sheaths where a concentration of 500 ng/ml was used. After mixing each sample, the fluorescence was measured at 454 nm (excitation wavelength 372 nm). Duplicate herringsperm DNA standards were prepared by incubating 1, 2, 3, 4 and 5 µl of a DNA standard solution (100 µg/ml) plus duplo blanks with 50 µl pronase solution. After incubation, 1 ml of a DAPI solution (conc. 50 or 500 ng/ml) was added and the fluorescence measured.

RESULTS

Fig.1A shows the fluorescence of the mithramycin-DNA complex as a function of the number of hair follicles from one person. The technique is shown to be sensitive for as little as one hair follicle. From the standard DNA curve (Fig.1B), it can be concluded that one hair follicle contains about 0.5 µg DNA. This is in accordance with the values for anagen scalp hairs obtained by the diphenylamine method (5).

Fig. 2A shows the fluorescence at 454 nm of the DAPI-DNA complex in various hair fractions. It can be concluded that the DNA from one single bulb can be estimated by means of the DAPI method. There is some degree of non-linearity, especially in the elongation zone, which can be attributed to variable cutting of the hair follicles into fractions and to individual hair root variations.

Comparison of the amount of DNA in the various fractions shows that the bulb comprises about 15%, the elongation zone 30% and the sheath 55% of the total amount of DNA in one hair follicle. The hair shaft contains only a residual quantity of DNA.

In a number of enzyme assays, which are currently performed with hair follicles, NADPH is used as a cofactor or is formed by the enzyme studied (6-8). This raises a problem with respect to

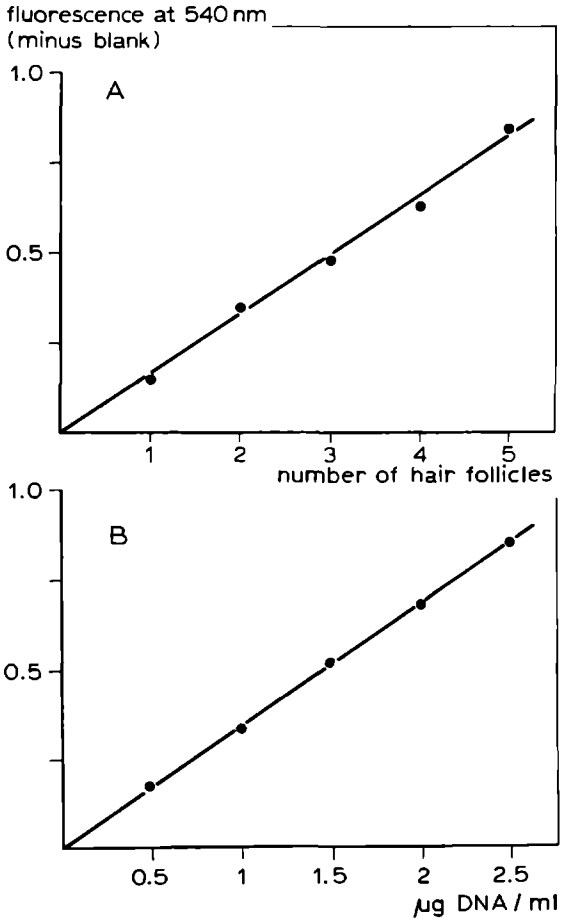

Fig. 1

(A) Fluorescence of the mithramycin-DNA complex of 1-5 hair follicles from one person. Five hair follicles give an increase in fluorescence of approx. 26% above the blank.
(B) Calibration curve for the fluorescence of the mithramycin-DNA complex.

Table 1 The influence of the glutathione-reductase system on the fluorescence of the DNA-DAPI complex.

incubation		relative fluorescence
enzyme system	DNA	
+	+	200
+	-	0
-	-	900
-	+	1100

All samples contained 1.7 mg NADPH/ml buffer (10mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 7.0). DNA was added to a final concentration of 5 µg/ml. The enzyme system consisted of 2 µmol glutathione (oxidized form) and 1.2 U glutathione-reductase.

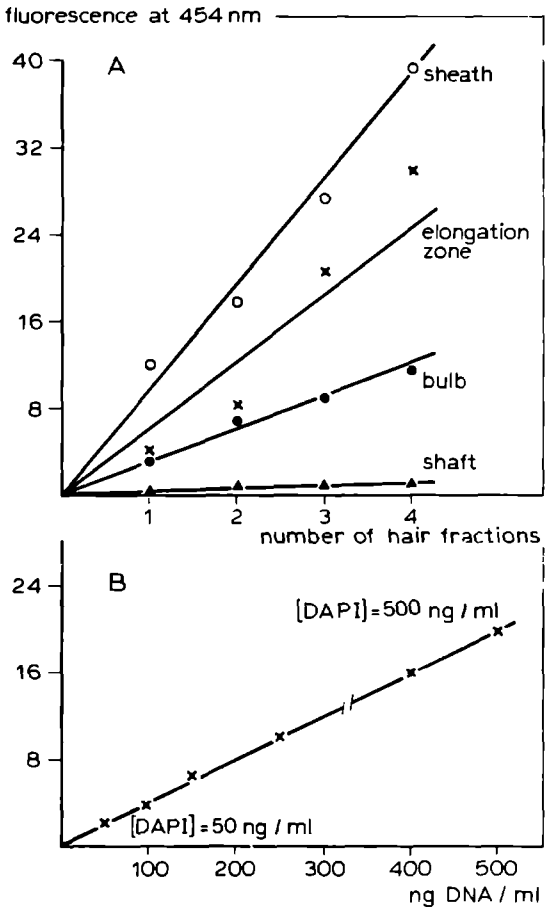

Fig. 2

(A) Fluorescence of the DAPI-DNA complex of 4 fractions of hair follicles from one person. Four bulbi give an increase in fluorescence of approx. 52% above the blank. For 4 elongation zones, sheaths and shafts, this value is 93%, 182% and 14% resp.
(B) Calibration curve for the fluorescence of the DAPI-DNA complex.

the DAPI method, because NADPH is highly fluorescent at the wavelength used (454 nm). In Table 1, we show that the interfering NADPH can be completely oxidized to NADP by means of the enzyme glutathione-reductase, using oxidized glutathione as a substrate.

DISCUSSION

The mithramycin method is based on the binding of the antibiotic to double-stranded DNA. This requires the disruption of cells to bring the DNA into solution. The recovery of DNA after cell lysis

by freezing and thawing, treatment with NaOH or sonification did not give reproducible results (data not shown). Treatment with a hypotonic pronase solution as described, however, seems to disrupt all hair follicle cells without interfering with the integrity of the DNA. In conclusion, the mithramycin method is very convenient for DNA determinations in one or a small number of hair follicles.

DNA determination in fractions of hair follicles was accomplished by using the specific intercalation of DAPI to DNA. From the results, it is clear that the DNA content of one single bulb can be accurately measured. From Fig. 2B, it can be calculated that the mean DNA amount in one bulb comprises about 75 ng, in the elongation zone 150 ng and in the sheath 275 ng. A complete hair follicle, therefore, contains about 500 ng DNA. This estimation is in accordance with the value obtained by the mithramycin method. It should be noted that the complex formation depends on the DNA : DAPI ratio and for this reason the DAPI concentration for the sheath fraction (which contains most DNA of the hair follicle) has been made ten times that used for the other fractions.

The present methods are advantageous for the microassay of DNA in a small number of hair follicles or in fractions of one single hair follicle. They offer the opportunity of comparative quantitation of biochemical parameters (e.g. activities of PAH metabolizing enzymes) in such small organs as hair follicles. Moreover, a reliable estimation of the number of viable cells can be made. Although primarily developed for the determination of individual differences of PAH metabolism in human hair follicles, the method may be useful for very small quantities of other tissue, e.g. skin specimens. Moreover, the DAPI method is very convenient for the use in cell cultures with very small numbers of cells ($<10^5$), especially for cultured hair follicle keratinocytes (11).

Application of the mithramycin method in a DNA test kit

The principle of liberating DNA from nucleoprotein complexes by pronase and the subsequent fluorescence determination of the mithramycin - DNA complex formed in the same test tube, renders this method particularly useful for routine application. Therefore we have developed - in close cooperation with Sanbio B.V., Nistel-

Fig. 3 Commercial DNA test kit as it has been developed from the method described in this chapter.

rode, The Netherlands - a DNA test kit with all necessary ingredients together in one small container. One package is enough for 60-120 determinations of DNA (including calibration curve samples). The method allows determination of DNA in cultured cells, skin biopsies, tumor specimens, hair follicles etc. The pronase supplied is now calibrated to an activity of 3000 U per ml instead of referring to weight (0.5 mg/ml) as described in this chapter. Fig. 3 shows the DNA test kit, which contains in one box: 2 ampoules dissolved DNA, 2 ampoules dissolved pronase, 2 ampoules desiccated mithramycin and one vial $MgCl_2$-solution for dissolving the mithramycin.

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4

Aluminium-Coated Cell for Fluorescence Signal Enhancement

ABSTRACT

Two methods for enhancement of the sensitivity in fluorescence spectrometry - a cell-holder with two concave mirrors and an aluminium-coated normal fluorescence cell - are described and compared to each other. Both devices result in an equal increase in sensitivity of the fluorescent signal. These techniques can be helpful e.g. in measuring biochemical parameters in very small samples.

INTRODUCTION

Fluorescence spectrometry is increasingly being used in chemistry. The reason is that it allows determination of chemical parameters with a higher sensitivity than the formerly used absorbance spectrometry (2,9). The tendency, for example in biochemistry, to measure parameters in smaller and smaller samples requires the use of very sensitive techniques (11). An improvement in the set-up that would lead to a higher sensitivity would be very helpful for the development of techniques for, for example, population studies that require the use of human biopsy tissue (4-6). In this chapter relatively simple designs are described that result in a marked improvement in fluorescence spectrometry. An example is given of an application where the increased sensitivity enables one to work with a smaller, acceptable amount of tissue specimens.

MATERIALS AND METHODS

Materials

Quinine hydrobromide (quinine-HBr) was obtained from Sigma (St. Louis, MO). For fluorometric measurements two spectrofluorometers were used: a Perkin-Elmer 3000 and a Perkin-Elmer 650-40. Fluorescent cells were purchased from Hellma GmbH & Co. (Müllheim-Baden, FRG). The cell holder device with two concave mirrors was obtained from Perkin-Elmer and was especially developed for the 650-40 Model. The coating of a fluorescent cell with aluminium was carried out as follows: after the fluorescent cell was thoroughly cleaned with Alconox and rinsed with alcohol and redistilled water, the cell was placed inside the coating chamber of an Edwards high vacuum coating unit, Model 12E6/579M (Crawley, U.K.). Two sides of the cell were covered to prevent disposition of aluminium onto these sides. The coating chamber was put under vacuum and aluminium of the highest purity was evaporated from a tungsten boat onto the rotating work holder containing the fluorescent cell. To prevent scratching of the aluminium coated cell a protective lacquer film was applied onto the cell and hardened in an oven.

Determination of signal/noise ratio

The following controls on the spectrofluorometer were selected as indicated: excitation wavelength 350 nm; emission wavelength 397 nm; excitation slit 10 nm; emission slit 10 nm; scan speed 60 nm/min. The emission spectrum of distilled water was recorded with the excitation wavelength set at 350 nm. The signal height of the Raman peak was then measured from the base line along a vertical to the peak (the maximal signal was reached at a wavelength of 397 nm). The spectrofluorometer was then prepared for time base measurements, and the noise level at the peak height of the Raman band (at 397 nm) was recorded.

Sensitivity of the fluorescent signal

A standard solution of quinine-HBr was used with a concentration of 2 ng/ml in 0.1 N H_2SO_4. The fluorescent signal was measured under the following conditions: excitation wavelength 350 nm; emission wavelength 450 nm; excitation slit 10 nm; emission slit 10 nm.

Sensitivity of the aryl-hydrocarbon hydroxylase assay

To test the usefulness of the cell device in a practical test, the enzyme aryl hydrocarbon hydroxylase was measured in human hair follicles. Collection of hair follicles and determination of the enzyme activity, based on the formation of phenolic benzo(a)pyrene metabolites, were performed as described earlier (5, see also chapter 5).

RESULTS AND DISCUSSION

The intensity of the fluorescent signal depends on several factors such as the intensity of the exciting radiation, the molar absorption of the sample at the excitation wavelength, the sample pathlength along the axis of irradiation, the concentration of

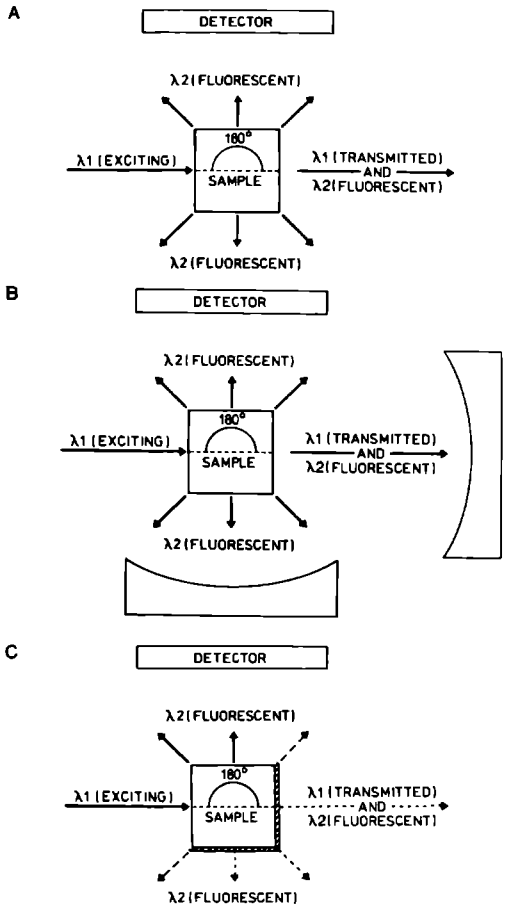

Fig. 1 (A) Normal cell. (B) Normal cell, placed in a cell holder containing two concave mirrors (opposite the excitation light source and opposite the detector of the emitted light). (C) Aluminium-coated cell. The two sides of the cell that are coated with an aluminium layer are the sides opposite the excitation light source and opposite the detector of the emitted light (heavy striped line). The dotted lines indicate the light that is reflected by the aluminium coating.

the fluorescing material and the geometry, depending on the effective angle viewed by the detector (8). Fig. 1 gives a schematic representation of the different cells used for improvement of the effective angle, that would result in a higher yield of fluorescent signal. Fig. 1A represents the traditionally used cell for

fluorescence measurements. In Fig. 1B the cell is placed in a holder containing two concave mirrors designed for the Perkin-Elmer 650-40 fluorometer. The mirror directly opposite the incoming excitation beam refocuses transmitted excitation light back onto the sample, thereby increasing the intensity of the exciting radiation. Light is emitted by the sample in all directions. The mirror directly opposite the emission slit reflects the emitted light, back to the emission collection optics, resulting in the increase of the fluorescent signal. In Fig. 1C the alternative device is demonstrated: two sides of a conventional cell are coated with an aluminium layer that reflects the light, thereby resulting in a higher yield of fluorescent signal.

The choise of aluminium as coating metal was based on the following considerations (1,3): the metals that are mostly used for the preparation of mirrors are aluminium and silver, since they give the highest reflection. In Fig. 2 the reflection characteristics of both materials over a wide range of wavelengths are compared. It can be seen that silver has the highest reflection in the visible range. However, silver mirrors rapidly deteriorate on

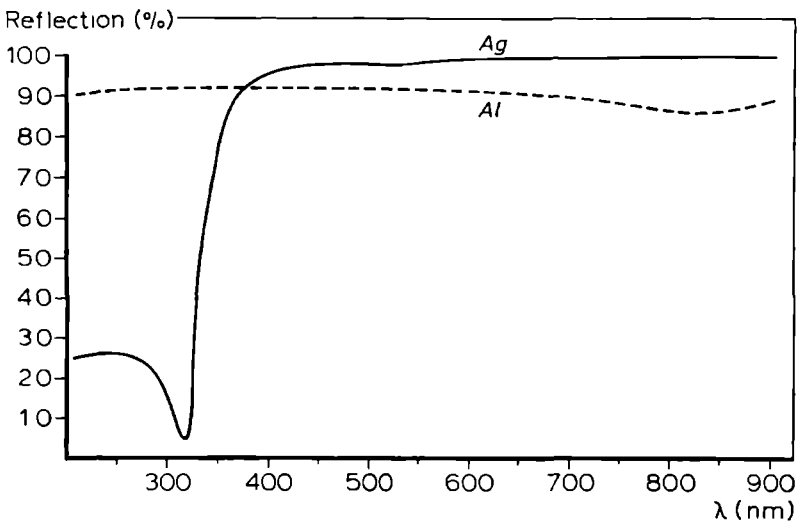

Fig. 2 Comparison of the reflection characteristics of aluminium and silver (adapted from ref. 1).

exposure in sulfur-contaminated atmospheres. Moreover, at a wavelength under 400 nm the reflection of silver falls abruptly, so that aluminium has to be used when UV light is employed. From Fig. 2 it can be concluded that aluminium has very good reflection characteristics from short-wave ultraviolet to the infrared region. Furthermore, aluminium is resistant to corrosion due to the very thin oxide layer that is formed immediately when aluminium comes into contact with air. This layer is very hard and resistant to chemicals, so that aluminium mirrors retain their high reflectivity over long periods of time. However, the thickness of this oxide layer is not sufficient to protect the underlying aluminium from mechanical damage. Therefore, a protective lacquer was applied. This protected the aluminium layer from scratches during handling, even after prolonged use.

Table 1 Determination of signal/noise ratio by use of the Raman band of distilled water [a]

fluorescence spectrophotometer	S/N ratio		
	I [b]	II [c]	III [d]
A. Perkin-Elmer 3000	26.0 ± 1.4 [e]		36.1 ± 1.9
B. Perkin-Elmer 650-40	87.6 ± 7.1	113 ± 9	109 ± 10

[a] *Excitation wavelength, 350 nm; emission wavelength, 397 nm; slit widths, 10 nm.* [b] *Normal cell.* [c] *Normal cell (cell holder with concave mirrors).* [d] *Aluminium-coated cell.* [e] *Results are expressed as mean ± standard deviation (n=5).*

The signal to noise ratio of the cells in the two fluorometers was determined by using the Raman band of distilled water. The results are shown in Table 1. A marked improvement in the signal to noise ratio is obtained with the use of the aluminium-coated cell and the cell with the mirror device as compared to the normal cell (note: the cell holder with the two concave mirrors was especially designed for the 650-40 model and could not be used in the other spectrofluorometer).

The effect of the different cells on the sensitivity of the fluorescent signal was determined by using quinine-HBr as a stan-

Table 2 Sensitivity of fluorescent signal[a]

fluorescence spectrophotometer	U_{fl}(max) I[b]	II[c]	III[d]	ratio II/I	III/I	III/II
A. Perkin-Elmer 3000	52.1±3.7		140.9±4.1		2.7	
B. Perkin-Elmer 650-40	86.7+4.5	223.4±13.7	222.7±9.2	2.6	2.6	1.0

[a]*As a standard a solution of quinine-HBr was used with a concentration of 2 ng/ml in 0.1 N H_2SO_4; excitation wavelength, 350 nm; emission wavelength, 450 nm; slit widths, 10 nm. The values given in the table were obtained after subtracting the blanks (mean ± standard deviation (n=5)).* [b]*Normal cell.* [c]*Normal cell (cell holder with concave mirrors).* [d]*Aluminium-coated cell.*

dard. The results of these measurements are summarized in Table 2. The fluorescent signal was improved by a factor 2.6-2.7 both using the aluminium-coated cell and the cell with the mirror device (as compared to the normal cell).

To test the usefulness of the aluminium-coated cell in a practical system, the activity of the enzyme aryl hydrocarbon hydroxylase was determined in human hair follicles. The enzyme activity in this human biopsy tissue might be related to individual cancer susceptibility in humans (10). For the determination of aryl hydrocarbon hydroxylase activity we used 30 hair follicles. The results are summarized in Table 3. When we define the determination limit (X_D) as a function of the standard deviation of the blank (σ_0) according to the formula $X_D = \bar{X}_0 + (k2^{\frac{1}{2}}\sigma_0/n^{\frac{1}{2}})$ (where $\bar{X}_0$ is the mean value of the blank and n is the number of blanks (7)), the enzyme could not be adequately measured in a normal cell.

Table 3 Aryl hydrocarbon hydroxylase assay[a]

	blank[b] (n=10)	hair follicles (n=5)	determination limit[c]
normal cell	2.44 ± 0.50	4.54 ± 0.36	4.68
normal cell (cell holder with concave mirrors)	4.19 ± 0.41	10.14 ± 0.81	6.03
aluminium-coated cell	4.35 ± 0.49	10.04 ± 0.71	6.54

[a]*Measurements performed with the 650-40 model.* [b]*Values expressed in units of fluorescence (mean±S.D.).* [c]*Calculated using $X_D = \bar{X}_0 + (k2^{\frac{1}{2}}\sigma_0/n^{\frac{1}{2}})$ (with k=10).*

However, with the mirror construction and the aluminium-coated cell, enzyme activity was above the determination limit. This example shows the usefulness of the both cells in population studies where high sensitivity with little biopsy material is required.

Although both the cell with the mirror construction and the cell with aluminium coating resulted in an equal increase in sensitivity of the fluorescence signal (Table 2 and Table 3), the cell with the mirror device has the disadvantage that it is especially developed for one type of fluorometer and cannot be used in other fluorometers. The cell with the aluminium coating is a universal design, that can be used in all types of fluorometers and is therefore much simpler to introduce in fluorescence spectrometry. In the next chapters (5-7) it will be shown that with the application of these devices, sensitive enzyme assays for aryl hydrocarbon hydroxylase, epoxide hydrolase and glutathione-transferase, especially suitable for hair follicles could be developed.

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5

Determination of Phenolic Benzo(a)pyrene Metabolites Formed by Human Hair Follicles

ABSTRACT

A modified fluorometric procedure for the determination of basal aryl hydrocarbon hydroxylase activity in human hair follicles is described. The method is also applicable for measurement of induction of the enzyme in primary cultures of keratinocytes originating from hair follicles. For good sensitivity of the assay the use of small incubation and extraction volumes, ultrapure chemicals, and adaptations in the measurement of the fluorescent signal are required. Moreover, a sensitive micromethod for measuring DNA as a reference variable has to be employed. The sensitivity of the assay permits the measurement of aryl hydrocarbon hydroxylase in 20 hair follicles or in the outgrowth of 3 cultured hair follicles with good reproducibility. The method gives the opportunity to identify individuals with genetically controlled differences in the metabolism of polycyclic aromatic hydrocarbons, using an easily obtainable biopsy tissue of epithelial origin.

INTRODUCTION

It has been recognized that polycyclic aromatic hydrocarbons (PAH) among which benzo(a)pyrene (BP) is the most extensively studied prototype compound, exert their mutagenic or carcinogenic action only after metabolic transformation to biologically active molecules (1,4,8,9,17). One of the enzyme systems responsible for the metabolism of PAH is aryl hydrocarbon hydroxylase (AHH), a mixed-function oxidase present in the endoplasmic reticulum of many tissues (11). Human hair follicles are a convenient tissue for

carcinogen metabolism studies in man, since they are of epithelial origin (most human malignancies arise in epithelia) and since they are easily removed from many volunteers without any risk. Therefore they could be applied in principle for population studies. It has been shown that interindividual variations in BP metabolism are partly determined by genetic factors, using human hair follicles (7) and cultured human lymphocytes (12) as biopsy tissue. Hitherto BP metabolism in hair follicles was analyzed by measurement of the radioactive 7,8- and 9,10-dihydrodiol metabolites of [^{3}H]BP (7,14). The fluorometric AHH assay as described by Nebert and Gelboin (10) was not sensitive enough for direct application to a limited number of hair follicles. Here we describe the modifications introduced in this AHH assay which enable the measurement of AHH levels in as few as 20 hair follicles or in the epithelial outgrowth of 3 cultured hair follicles.

MATERIALS AND METHODS

Collection of hair follicles

Hair follecles were plucked from random areas of the scalp of healthy volunteers (nonsmokers). Only hair follicles with visible bulb and sheath were used.

AHH assay

The assays were performed in triplicate. For each assay 20 hair follicles were immersed in 0.2 ml incubation medium in Eppendorf (Hamburg, FRG) reaction vials. The incubation medium consisted of 50 mM Tris-HCl, pH 8.5, containing 0.1 M sucrose, 2 mM NADPH (Boehringer-Mannheim, FRG), 3 mM $MgCl_2$, gentamycin (10 µg/ml) (Schering, Berlin, FRG). Two microliters of a stock solution of BP (Aldrich, Beerse, Belgium) of a concentration of 6.2 mg/ml dissolved in a mixture of ethanol and dimethylsulfoxide (1:1, v/v) was added. After careful mixing, the reaction vials were placed with opened lids in a shaking water bath and incubated for 1 h in the dark at 37°C. The reaction was stopped by adding 0.2 ml of ice-cold acetone (Uvasol for fluorescence spectroscopy, Merck, Darmstadt, FRG). After mixing the samples, 0.8 ml of n-hexane (Uvasol) was added and the vials were placed in an Eppendorf shaker for 5 min. The phases were separated by centrifugation for 1 min in an Eppendorf minifuge. The organic upper phase was collected in a glass tube and put on ice. Then 0.8 ml of 1 N NaOH (Suprapur, Merck) was added and the tubes were thoroughly vortexed during 30 s. The phases were separated by centrifugation at 6000g during 5 min at 0°C. The organic phase was removed completely and discarded. The amounts of extracted hydroxylated BP derivatives were immediately measured in a spectrofluorometer (Perkin-Elmer 650-40) at a wavelength of 396 nm and an emission wavelength of 522 nm. To amplify the fluorescent signal a cell holder containing two concave mirrors was used (Fig.1 chapter 4; see Results and Discussion). 3-Hydroxybenzo(a)pyrene, kindly provided by the National Cancer

Institute Chemical Repository at the IIT Research Institute, Chicago, Illinois, was used as a standard. In routine experiments, quinine HBr in 0.1 N H_2SO_4 was used for calibration of the spectrofluorometer.

Culturing of hair follicle cells and induction of AHH

Human hair follicle cells were cultured on an extracellular matrix (bovine eye lens capsules (5))using an especially constructed culture dish (Epicult, provided by Sanbio, Nistelrode, The Netherlands; see also chapter 2) as described earlier (16). AHH was induced in the cultures by exposure to culture medium (minimum essential medium, Gibco, Glasgow, U.K., supplemented with 15% heat-inactivated serum, 2 mM glutamine, and 10 µg/ml gentamycin sulfate) containing 10 µM benz(a)anthracene in dimethylsulfoxide (DMSO). Control cultures were treated with DMSO alone. The final DMSO concentration in induced and control cultures was 0.1%. The hydrocarbon exposure was carried out for 16 h. After exposure the cultures were rinsed four times with 0.9% NaCl solution. The AHH-assay was carried out as described for freshly isolated hair follicles, except that the incubation was performed in a volume of 2 ml during 2 h in Falcon culture flasks. For this purpose the outgrowth in three Epicult dishes (each containing one hair follicle) was combined. Extraction volumes were adapted to the increased volume: the reaction was stopped with 1 ml acetone, organic-solvent soluble metabolites of BP extracted with 3 ml hexane and hydroxylated BP metabolites extracted with 3 ml 1 N NaOH.

Determination of DNA

DNA in the hair follicles and cultures of hair follicle keratinocytes was measured by the mithramycin technique (3) with modifications according to Hukkelhoven et al. (6; see also chapter 3). Enzyme activities were expressed as picomoles phenolic metabolites per microgram DNA per hour.

RESULTS AND DISCUSSION

Characterization of the assay

Comparison of the excitation and emission spectra of the alkali-extracted metabolites of BP and standard 3-hydroxy-BP reveals that the fluorescence is largely attributable to hydroxylated BP derivatives (Fig.1). Although other BP metabolites are also formed by hair follicles, especially dihydrodiols (7,14), they are either not extracted into the alkaline phase or, when partially extracted, do not interfere with the phenol assay, since they contribute relatively insignificant fluorescence compared to the phenols (18). Further evidence that we are dealing with mixed-function oxidase activity in human hair follicles is provided by the observations that addition of 5 mM EDTA and exposure to nitrogen leads to a strong reduction in fluorescence (Table 1), reflecting the

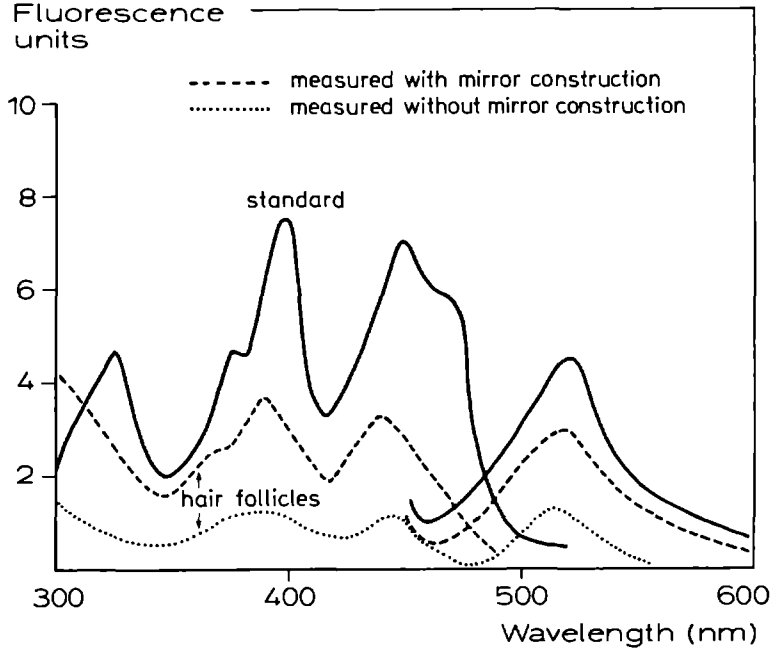

Fig. 1 Excitation (left) and fluorescence (right) spectra of 3-hydroxybenzo-(a)pyrene standard solution and extracted hydroxylated BP derivates from 20 hair follicles measured with and without the mirror construction in the fluorometer (see chapter 4). Excitation slit width is 5 nm, emission slit width is 10 nm.

Table 1 AHH activity in 20 hair follicles using different incubation systems

incubation system	relative fluorescence
incubation as described	100
+5 mM EDTA	5
incubation in atmosphere of N_2	9
-BP	2
-NADPH	79
incubation at 0°C	3
boiled hair follicles	7

need for divalent cations and molecular oxygen. However, the reaction has only a limited requirement for exogenously administered NADPH. This can be due to production of relatively high intracellular levels of NADPH in hair follicle cells. The presence of high levels of glucose-6-phosphate dehydrogenase, the key enzyme of the pentose-phosphate cycle, has been demonstrated in hair follicles (15). The enzymic nature of the reaction is illustrated by the absence of hydroxylation at 0°C and in boiled hair follicles. The very low blank values obtained when incubations are performed without hair follicles, or with hair follicles at 0°C, indicate that negligible amounts of BP are extracted with the hydroxylated BP-metabolites during the NaOH-extraction step.

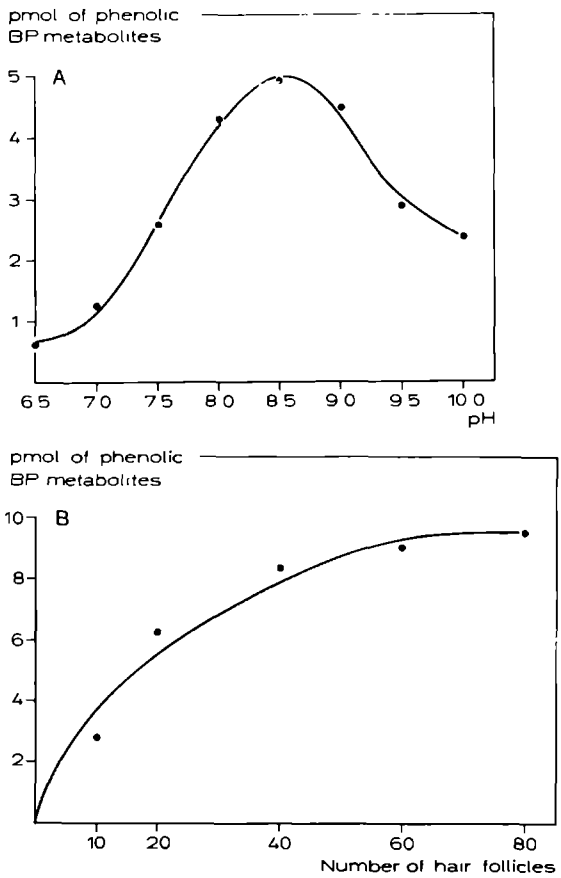

Fig. 2 (A) Effect of pH on AHH activity in 20 hair follicles.
(B) Effect of the number of hair follicles on AHH activity.

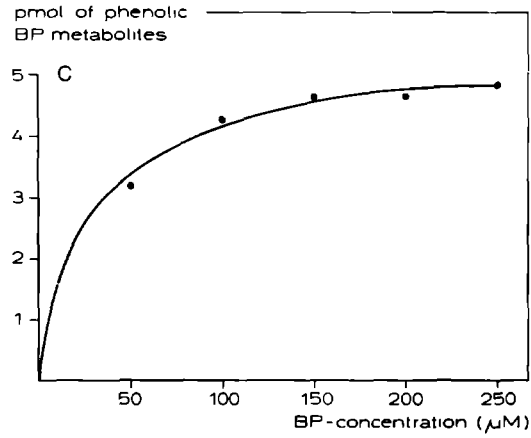

Fig. 2 (C) Effect of substrate concentration on AHH activity in 20 hair follicles.

Assay conditions

The formation of fluorescent metabolites of BP by human hair follicles is affected by the pH of the incubation medium (Fig. 2A), the number of hair follicles (Fig. 2B), the substrate concentration (Fig. 2C), and the time of incubation. Maximal enzyme activity was found at pH 8.5. In order to arrive at a reasonable number of hair follicles to be used in population studies, 20 hair follicles were used in each incubation. The concentration of BP needed to reach maximal enzyme velocity was 200 μM. This relatively high value can be explained by substrate depletion due to binding to nonspecific sites in the hair follicles and by slow diffusion of the substrate through the keratin layers of the hair follicles. For routine experiments an incubation time of 1 h was chosen. Assays from a limited number of volunteers (25) have shown that basal AHH activities vary between 0.1 and 1.0 pmol phenolic metabolites/μg DNA/h. When cultures of primary human keratinocytes originating from hair follicles were preexposed to benz(a)anthracene an increased BP metabolism toward phenolic products could be observed. This AHH-induction phenomenon has been described in a variety of tissues of different species and in many cells grown *in vitro* (2,11). Although we have not yet performed enough AHH-induction

assays on cultured keratinocytes to have a reasonable idea of induction figures, the few persons that were measured showed induction ratios (induced activity/basal activity) of around 3. Basal activities in culture and in freshly isolated human hair follicles were quantitatively comparable. The use of very pure chemicals in the extraction procedure was essential in order to reduce blank values to appropriate limits.

Quantitating activity

For quantitative expression, AHH activity is often defined as the amount of phenolic metabolites per unit of (microsomal) protein. Since in hair follicles much protein is associated with nonliving keratinized cells, measurement of DNA as a reference variable is an attractive alternative. The micromethod of DNA measurement which we employ for this purpose (6) involves treatment of the hair follicles with a hypotonic pronase solution resulting in solubilization of DNA, which is then available for complex formation with mithramycin. Using DNA as a reference variable AHH activity in hair follicles can be measured with good reproducibility (see also chapter 3).

Measurement of the fluorescent signal

The quantitative relationship between fluorescence intensity and low concentrations of fluorescing material has been described (13) as follows: $(Sf)\lambda = f(\theta)g(\lambda)I\phi fabc$, where

$(Sf)\lambda$ is the sample fluorescence intensity at a given wavelength

$f(\theta)$ is the geometry depending on the effective angle viewed by the detector

$g(\lambda)$ is the response characteristic of the detector (varies with wavelength)

I is the intensity of the exciting radiation

ϕf is the quantum efficiency of the molecules

a is the molar absorption of the sample at the excitation wavelength

b is the sample pathlength along the axis of irradiation

c is the concentration of the fluorescing material in moles per liter.

(This relation is only valid if abc for the sample is less than 0.05).

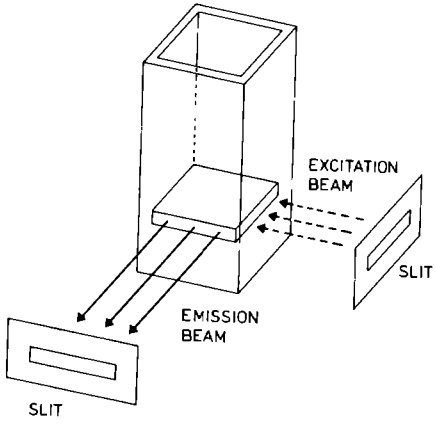

Fig. 3 Light path in the horizontally oriented entrance slit.

In the model 650-40 light passes through a horizontal slit (Fig. 3) which improves $f(0)$ and therefore $(Sf)\lambda$. A second advantage of the horizontal light beam, positioned very near to the bottom of a cuvette, is the fact that the required sample volume is only 0.6 ml in a standard 1 cm pathlength cuvette. This results in improved sensitivity per sample volume. For further improvement of the detection limit we used a high-sensitivity cell holder which incorporates two concave mirrors on two sides of the cell holder. Technical details of this device are described in chapter 4 of this thesis. By means of this acessory a theoretical fourfold increase in $(Sf)\lambda$ results in practice in a sensitivity improvement by a factor of 2.6 as measured from the signal-to-noise ratio using the Raman band (excitation wavelength,350 nm; emission wavelength, 397 nm) of distilled water.

Conclusion

In the present study we have shown that AHH can be measured adequately in as few as 20 hair follicles or in the outgrowth of only 3 cultured hair follicles. The described assay offers the opportunity to identify individuals with genetically controlled differences in carcinogen metabolism. With the assay basal AHH

levels can be determined in freshly isolated hair follicles and AHH inducibility can be measured using cultured human hair follicle keratinocytes.

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6

A Sensitive Fluorometric Assay for Epoxide Hydrolase

ABSTRACT

A rapid and sensitive fluorometric assay for epoxide hydrolase, using 4,5-dihydro-epoxybenzo(a)pyrene as substrate, is described. The method is especially convenient for measurement of the enzyme activity in small extrahepatic tissue samples exhibiting a low specific activity of epoxide hydrolase. The experimental procedure combines a simple 2-step extraction procedure with the sensitive fluorometric determination of the product trans-4,5-dihydrodiol-benzo(a)pyrene. The method is especially developed for the use of human hair follicles, a tissue suitable for investigations concerning the relationship between activities of carcinogen metabolizing enzymes (among which epoxide hydrolase) and genetic susceptibility to carcinogen-induced neoplasia.

INTRODUCTION

It is now firmly established that a great number of xenobiotics, e.g., drugs, environmental pollutants and food additives can be metabolized by microsomal cytochrome P450-dependent monooxygenases to highly reactive epoxide intermediates (8,17). Epoxide hydrolase (EC 3.3.2.3.) has a dual role in the further metabolism of these epoxides. On the one hand, hydration of arene oxides to the chemically less reactive trans-dihydrodiols can be considered as a detoxification pathway, while on the other hand certain trans-dihydrodiols of polycyclic aromatic hydrocarbons (PAH) can be further oxidized by the monooxygenases to highly mutagenic and

carcinogenic diol-epoxides (2,18).

Since inter-individual differences in carcinogen metabolism can result in different susceptibility to obtain carcinogen-induced neoplasm, it is of interest to detect genetic differences in activity of the various carcinogen metabolizing enzymes (1,10). Human hair follicles have been suggested as a convenient biopsy-tissue for these investigations (7,19). Here, we present a rapid and very sensitive fluorometric assay for epoxide hydrolase (EH), using 4,5-dihydro-epoxybenzo(a)pyrene as substrate, which is especially useful for hair follicles and for other small tissue samples with relatively low EH activities.

MATERIALS AND METHODS

Chemicals

4,5-Dihydro-epoxybenzo(a)pyrene and trans-4,5-dihydrodiolbenzo(a)pyrene were obtained from the National Cancer Institute Chemical Repository (IIT Research Institute, Chicago, IL). Trichloropropylene oxide (TCPO) was purchased from Aldrich (Beerse, Belgium).

Biopsy material

Human hair follicles were obtained from the scalp of normal volunteers (nonsmokers). Only hair follicles with visible bulb and sheath were used. Human lung specimens, macroscopically free of tumor tissue, were obtained from lung resection operations. After coarsely mincing the tissue with scissors and rinsing in several changes of homogenization buffer (0.1 M potassium phosphate (pH 7.9), 10% (v/v) glycerol and 1 mM EDTA), 20 g material was homogenized, first in a Waring blender (20 s at maximal speed) and then in a Potter S homogenizer (Braun). The homogenate was centrifuged at 10,000g for 1 h and 1/2 of the resulting supernatant was used for preparation of microsomes by centrifugation at 120,000g for 1 h. The microsomal pellet was resuspended in 0.1 M Tris (pH 9.5). Protein in the 10,000g supernatant and microsomes was adjusted to approximately 5 mg/ml.

Assay procedure

All assays were performed in triplicate. For each assay 15 hair follicles or 50 µl lung 10,000g supernatant or microsomes were added to 0.5 ml final vol. 0.1 M Tris (pH 9.5). The reaction was initiated by addition of the substrate in 10 µl dimethylsulfoxide (DMSO) (final conc. 100 µM) and carried out in a shaking water bath at 37°C. After the required incubation time, the reaction was stopped by addition of 3.5 ml petroleum ether (b.p. 40-60°C), shaking the tube on a Vortex mixer for 5 s and cooling in ice. Then 0.5 ml of DMSO was added and the tubes were mixed on a Vortex mixer for 60 s. After centrifugation at 5,000g for 2 min the petroleum ether fraction was removed and the extraction procedure repeated twice, resulting in removal of the unreacted substrate (16). The product, trans-4,5-dihydrodiolbenzo(a)pyrene, was then extracted into 1 ml ethylacetate by agitating on a Vortex mixer for 90 s. After separation of the

phases by centrifugation at 5,000g for 5 min the ethylacetate was transferred to a new tube and the fluorescence measured in a Perkin-Elmer 650-40 spectrofluorometer at an excitation wavelength of 312 nm and an emission wavelength of 388 nm. The spectrofluorometer was equiped with a cell holder containing two concave mirrors (see chapter 4). Known amounts of trans-4,5-dihydrodiolbenzo(a)pyrene were used as standards. In routine experiments, quinine HBr in 0.1 N H_2SO_4 was used for the calibration of the fluorometer. DNA in the hair follicles was measured by the mithramycine technique (6) employing calf thymus DNA as a standard and protein in the lung samples was determined as in (9) using bovine serum albumin as a standard. Enzyme activities were expressed as pmol trans-4,5-dihydrodiolbenzo(a)pyrene/µg DNA/h and in the lung samples as nmol trans-4,5-dihydrodiolbenzo(a)pyrene/mg protein/h.

RESULTS

Since the assay has been primarily developed for use of human hair follicles, optimal assay conditions have been determined for this tissue type. Fig.1 shows the excitation and fluorescence spectra of pure trans-4,5-dihydrodiolbenzo(a)pyrene and the ethylacetate fraction after the enzyme reaction using 15 hair follicles. Comparison of both spectra shows that the fluorescence in the extract is almost entirely due to trans-4,5-dihydrodiolbenzo(a)-pyrene.

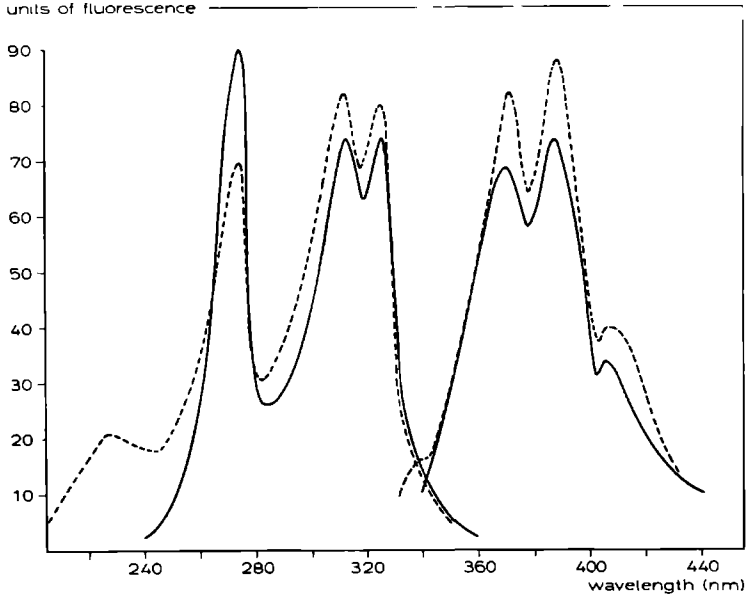


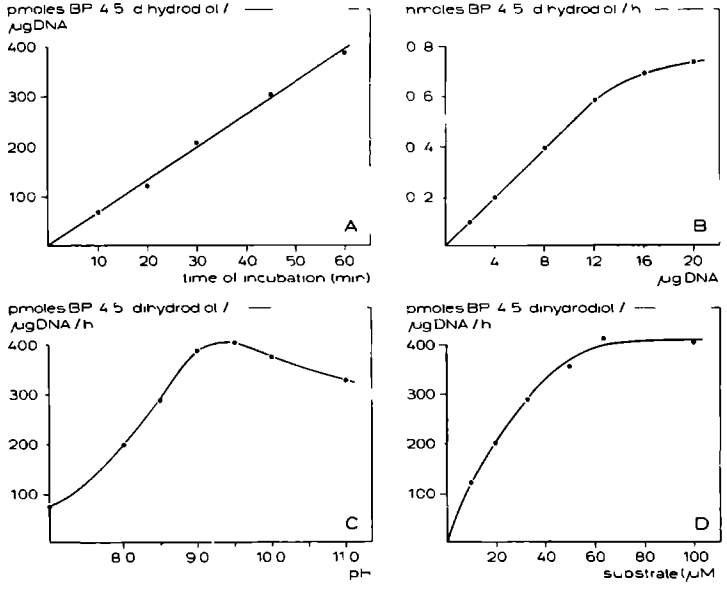
Fig. 1 Excitation (left) and fluorescence (right) spectra of pure trans-4,5-dihydrodiolBP (dotted lines) and the ethylacetate extract after the enzymatic reaction using 15 hair follicles (solid line).

Addition of TCPO (which has been described as a potent uncompetitive inhibitor of hepatic EH (12)) at a final concentration of 1.8 mM results in almost complete inhibition of enzyme activity in human hair follicles (Table 1). The enzymic nature is further demonstrated by the absence of hydration in boiled hair follicles and at 0°C.

Table 1 EH levels in various incubation systems

samples	activity
human lung	
10,000 x g supernatant[a]	10.9
microsomes[a]	72.6
human hair follicles[b]	440
+ 1.8 mM TCPO[b]	33
- 4,5-dihydro-epoxyBP[b]	0
incubation at 0°C[b]	0
boiled[b]	10

[a] *Activity expressed as nmol 4,5-dihydrodiolBP/mg protein/h*
[b] *Activity expressed as cmol 4,5-dihydrodiolBP/µg DNA/h*
Assays were performed as in Materials and Methods.



The reaction is linear with time for at least up to 60 min and with DNA-concentration up to 12 µg, corresponding to about 25 hair follicles (Fig. 2A,B). Since with 15 hair follicles enzymatic rates are 3-6 times as high as those of the blank without enzyme, a number of 15 hair follicles has been used routinely. Optimal enzyme activity was reached at pH of 9.5 (Fig. 2C). Substrate concentrations exceeding 100 µM did not result in any increase in enzyme activity (Fig. 2D). From a Lineweaver-Burk plot (Fig. 3) the K_m and V_{max} can be determined for EH in hair follicles. The app.K_m is 23 µM, a value higher than that for lung microsomes (8 µM) which could be caused by substrate depletion due to binding to non-specific sites in the hair follicles.

Using human lung preparations the reaction proceeds linearly with time for 20 min with microsomes (0.5 mg protein/ml) and for 60 min with 10,000 x g supernatant (0.5 mg protein/ml). With a standard incubation time of 30 min linearity with protein is maintained up to 0.6 mg protein/ml with lung microsomes and up to 1.3 mg protein/ml with lung 10,000 x g supernatant.

DISCUSSION

A wide range of assays for EH have been described including radiometric (14), photometric (20), gas chromatographic (5) and liquid chromatographic assays (15). Each of these assays has its own limitations for application in large population studies such as the use of radioactive substrates, low sensitivity (photometric assay), the need for derivatization (gas chromatography) or tedious procedures (liquid chromatography). The continuous fluorometric assay developed in (4), although very sensitive, is limited by the

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Fig. 2 Kinetics of the hydration of 4,5-dihydro-epoxyBP by human hair follicles. All data are the mean of triplicate incubations: (A) time-velocity relation (using 15 hair follicles corresponding to approximately 7.5 µg DNA; 100 µM substrate); (B) DNA-velocity relation (60 min incubation; 100 µM substrate); (C) pH-velocity relation (60 min incubation; 15 hair follicles; 100 µM substrate); (D) substrate-velocity relation (60 min incubation; 15 hair follicles).

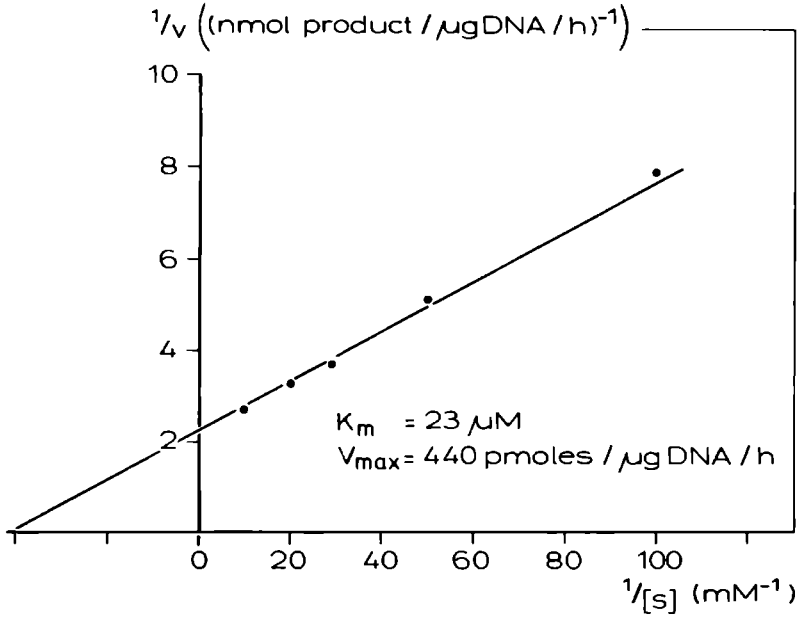

Fig. 3 Double-reciprocal plot of the variation of the rate of enzymatic 4,5-dihydro-epoxyBP hydration as a function of substrate concentration. Triplicate assays were performed with 15 hair follicles and by incubation for 60 min.

fact that at higher substrate concentrations (>10 µM) the absorbance of the solution is not sufficiently low to avoid quenching and by the photodecomposition of substrate and product. The fluorometric endpoint assay described here combines the efficient separation of substrate and product with the sensitive fluorometric determination of the product in the ethylacetate fraction. In the assay, EH is measured using an epoxide of PAH, a class of chemicals in which the crucial role of EH in activation and inactivation of biologically active intermediates, is obvious. Moreover, 4,5-dihydro-epoxyBP has a relatively very low spontaneous rate of hydrolysis. Although we have used 4,5-dihydro-epoxyBP obtained as a gift from the National Cancer Institute, the compound can be synthesized relatively easily from BP (3). The K_m-values obtained with this substrate (8 µM for human lung microsomes and 23 µM for human hair follicles) are much lower than those reported for, e.g. styrene oxide, using human and guinea pig liver preparations

(11,13).This is consistent with the idea that EH has evolved as a response to the continuous presence of PAH as a result of incomplete combustion. The described assay offers the opportunity to investigate the possible interrelationship between EH levels and susceptibility to develop cancer upon exposition to PAH.

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7

A Highly Sensitive Assay for Glutathione Transferase Using 4,5 - Dihydro-epoxybenzo(a)pyrene as Substrate

ABSTRACT

A method is described for the quantitative determination of the glutathione conjugate of 4,5-dihydroepoxybenzo(a)pyrene. The sensitivity and practical convenience of the procedure is based on (a) the high specific fluorescence intensity of the product, (b) the very low background obtained by the efficient differential extraction of substrate and product, (c) the use of a non-radioactive substrate from the important class of polycyclic aromatic hydrocarbons and (d) the involvement of a single rapid transfer and extraction step. Due to the sensitivity of the method (permitting measurement of 30 pmoles product) the procedure is especially useful for assaying transferase activity in minute tissue samples such as human hair follicles or cultured cells.

INTRODUCTION

Arene oxides - which arise from the enzymatic oxydation of polycyclic aromatic hydrocarbons (PAH) - are electrophilically reactive agents which can alkylate biological nucleophiles (e.g. DNA) leading to toxic, mutagenic or carcinogenic effects (11,17). Besides their importance as direct noxious substances, they are - after hydration to dihydrodiols - the precursors of the ultimate carcinogens of PAH, the diol-epoxides (10). Therefore it is clear that understanding of the degradation of these compounds is important for an appreciation of their fate and action in biological systems.

Epoxides can be metabolized by epoxide hydrolase (EH; EC 3.3.2.3) yielding the already mentioned dihydrodiols or by glutathione transferase (GSH-T; EC 2.5.1.18) catalyzing the nucleophilic attack of the sulfur anion of glutathione (GSH) on the epoxide, leading to the corresponding conjugate. Whereas the effect of EH can be considered as either toxification - by providing the precursors of the diol-epoxides - or detoxification - by providing a possible conjugative pathway of sulfotransferase action on the dihydrodiols - the GSH-T action is generally considered to act as detoxification of the parent compound. It has been found recently that one form of glutathione-transferase is also capable of inactivating diol-epoxides of PAH (5). In the present study we describe a fluorometric assay for the conjugation of 4,5-dihydroepoxybenzo(a)pyrene (BPO) with GSH. The method has several advantages over earlier published assays for this enzyme, such as the use of a non-labeled substrate of an important class of chemical carcinogens (PAH), the high sensitivity of the method permitting measurements in extrahepatic tissues or cultured cells, and the rapid and simple assay procedure.

MATERIALS AND METHODS

Chemicals

BPO was obtained from the National Cancer Institute Chemical Repository at the IIT Research Institute, Chicago, Ill. 17-β-Estradiol-3-monosulfate was purchased from Sigma, St. Louis, Mo. and reduced GSH from Boehringer Mannheim, Mannheim, FRG. L-glycine-2-^{3}H-glutathione (reduced; specific activity 1 Ci/mol) was obtained from New England Nuclear, Dreieich, FRG.; t.l.c.-plates (silica gel 60, without fluorescence indicator) were from Merck, Darmstadt, FRG.

Source of enzyme

Liver tissue was obtained from Wistar rats. After coarsely mincing the tissue with scissors the liver was homogenized in buffer (0.1 M potassium phosphate (pH 7.9), 10% (v/v) glycerol and 1 mM EDTA) using a Potter S homogenizer (Braun). The homogenate was centrifuged at 10,000 x g for 1 h at 4°C. The resulting supernatant was used for preparation of the cytosol by centrifugation at 105,000 x g for 1 h at 4°C. The protein content of the supernatant was determined by the method of Lowry et al. (12) with bovine serum albumin as the reference standard.

Assay procedure

All assays were performed in triplicate. For each assay 3 µl liver supernatant (representing 5.5 µg protein) and 10 µl GSH (20 mM) were added to 0.5

ml final volume 25 mM glycine (pH 10.0) in an Eppendorf reaction vial. The vials were pre-warmed for 5 min in a shaking water bath at 37°C. The reaction was initiated by addition of 4 µl of substrate dissolved in DMSO (final BPO concentration 15 µM) and carried out in the water bath at 37°C. After the required incubation time (standard 5 min) the reaction was stopped by adding 0.5 ml trichloroacetic acid (TCA; 7.5% w/v). After centrifugation for 2 min in an Eppendorf minifuge the reaction mixture was transferred to a glass tube and put on ice. To remove the unmetabolized BPO, 3 ml of hexane was added and the tubes were mixed on a Vortex mixer for 60 sec. After centrifugation for 5 min at 5,000 x g, the hexane fraction was removed and 0.5 ml aqua bidest added to the water phase. The fluorescence was measured in a Perkin Elmer 650-40 spectrofluorometer at an excitation wavelength of 305 nm and an emission wavelength of 425 nm. Quinine HBr in 0.1 N H_2SO_4 was used for calibration of the fluorometer.

Calibration curve of the conjugate

Quantification of the adduct was performed by overnight t.l.c. separation of the reaction mixture as described by Nemoto and Gelboin (14) using ^{3}H-labeled GSH (0.3 µCi/incubation), diluted with GSH to the standard concentration of 400 µM. The reaction was stopped by putting the reaction vials on ice instead of adding TCA. The area of the conjugate (R_f = 0.15) was scraped off the plate and the conjugate isolated by extraction with water. A sample of the extract was analyzed for radioactivity and the amount of conjugate formed was calculated from the specific activity of the compound. Fluorescence was correlated to the amount of conjugate by preparing dilutions of conjugate-extracts obtained simultaneously under standard conditions (i.e. without ^{3}H-GSH). Enzymatic activities were expressed as pmoles conjugate/µg protein/min. This establishment of the relation between units of fluorescence and amount of conjugate has to be performed only once.

RESULTS

Fig. 1 shows the excitation and emission spectra of the BP-GSH conjugate isolated from t.l.c. and from the aqueous phase after the enzymatic reaction using rat liver supernatant. The similarity of the maximum excitation and emission wavelengths of both products shows that the fluorescence in the aqueous phase results almost entirely from the BP-GSH conjugate. Addition of 17β-estradiol-3-monosulfate, which has been described as a selective inhibitor of several forms of GSH-T (15), results in a 25% decrease of enzyme activity. Hg, for which an inhibitory role, among other heavy metals, of GSH-T *in vitro* has been documented (4), results in about the same percentage of enzyme inhibition. The enzymic nature of the reaction is further demonstrated by the low rate of conjugation using boiled supernatant, at 0°C and in the absence of BPO.

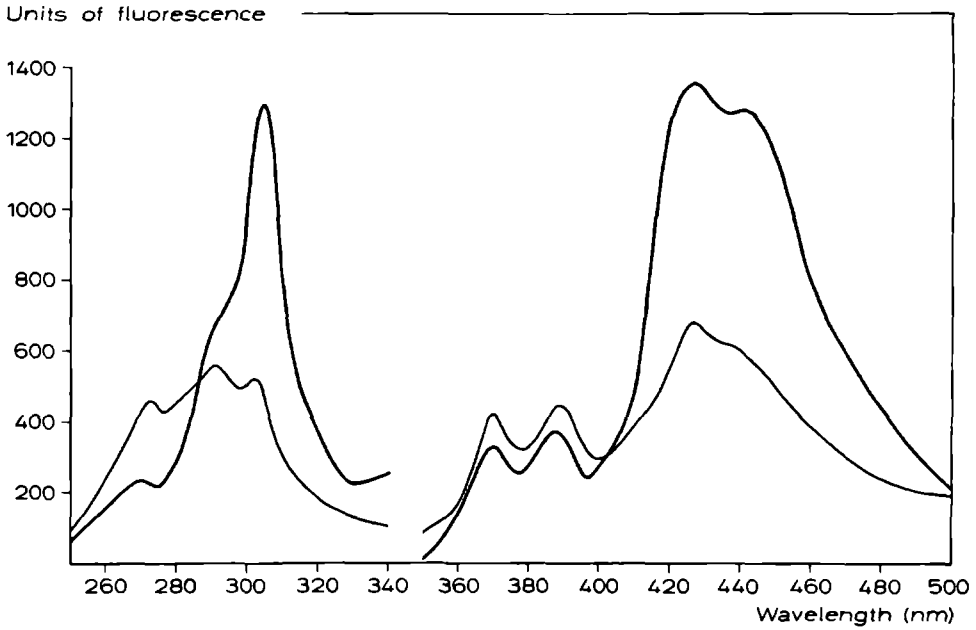

Fig. 1 Excitation (left) and emission (right) spectra of BP-GSH isolated from t.l.c. as in ref. 14 (light lines) and from the aqueous phase after the enzymatic reaction using rat liver 105,000 x g supernatant as in Materials and Methods, section 'Calibration curve of the conjugate' (heavy lines).

From Fig. 2 it can be concluded that linearity is maintained up till about 7 min of incubation (A) and up till 7 µg protein (B). The pH optimum of the reaction is reached at pH 10 (Fig. 2C). The non-enzymatic addition of GSH to BPO is strongly pH-dependent and is particularly manifest at pH > 10. Substrate concentrations exceeding 10 µM did not result in higher enzymatic rates (Fig. 2D). The K_m of the reaction is about 2.3 µM and the specific activity of the enzyme 84 pmol/µg protein/min as can be derived from Fig. 2D.

The assay was also performed with freshly isolated hair follicles under standard conditions. It was found that glutathione conjugation to BPO could be adequately measured in one single hair follicle. Inhibition of the reaction by 17β-estradiol-3-monosulfate and Hg, as well as absence of conjugation in boiled hair follicles, at 0°C and in the absence of substrate, were comparable to that

Table 1 The properties of GSH-T

samples	activity[a]
complete	100
+ 17ß-estradiol-monosulfate (50 µM)	75
+ $HgCl_2$ (37 µM)	76
boiled liver supernatant	12
incubation at 0°C	11
- BPO	0

[a] *Activity is expressed as a percentage of the value obtained with the standard procedure as described in Materials and Methods, section 'assay procedure'.*

found with rat liver supernatant.

It should be noted that the recording of the optimal signal of the fluorometer is delayed. Although the exact reason for this phenomenon is not known, we suppose that dehydration of the conjugate at the low pH (TCA) and in the presence of UV radiation, results in the formation of a compound with a more aromatic character and therefore increased fluorescence. After about 3 min the fluorescence signal is stabile and can be read. Addition of TCA after the enzymic reaction is very convenient since it not only terminates the conjugation due to the pH shift to pH 2 but also results in precipitation of proteins and a higher specific fluorescence intensity of the conjugate.

DISCUSSION

A number of assays for GSH-T has been described including titrimetric, colorimetric, spectrophotometric and radiochemical methods using a variety of substrates (for a review see 2). However, assays using substrates of the biologically important PAH have only employed radioactive substrates (1,6,13,14), most of them using a time consuming t.l.c. separation of the conjugate. In addition,

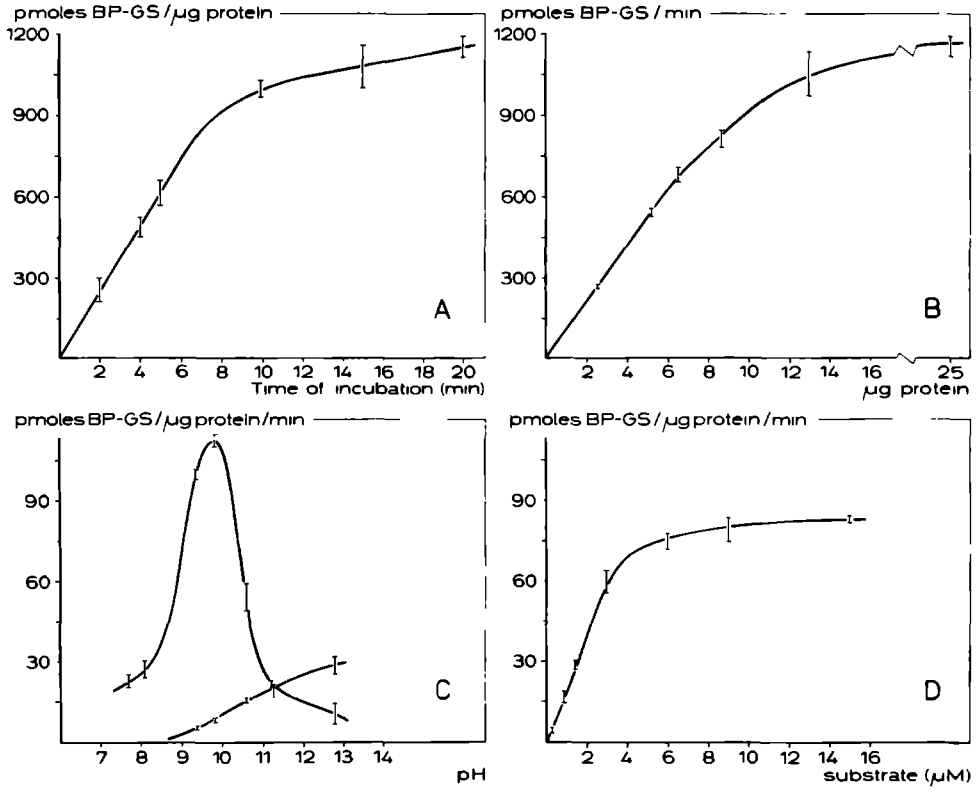

Fig. 2 Kinetics of the enzymatic conjugation of BPO to GSH by rat liver supernatant. All data are expressed as mean (n=3) ± S.D. and are obtained by subtraction of the non-enzymatic blanks from the sample values.
(A) time-velocity relation (3 µl supernatant corresponding to 5.5 µg protein; 15 µM substrate; pH 10.0).
(B) protein-velocity relation (5 min incubation; 15 µM substrate; pH 10.0).
(C) pH-velocity relation (5 min incubation; 5.5 µg protein; 15 µM substrate).
(D) substrate-velocity relation (5 min incubation; 5.5 µg protein; pH 10.0).

enzyme activity is often measured at suboptimal pH due to the large contribution of the non-enzymatic conjugation. The present method combines an extreme sensitivity with the easy separation of the conjugate of glutathione with a non-radioactive PAH substrate. Optimal conditions (substrate, enzyme and glutathione concentrations) were obtained at 1/5 of those described by Van Cantfort et

al. (1) who have used the same extraction principle. pH-Optimum, time-dependence and non-enzymatic conjugation were quantitatively comparable between the former assay and the present one. Although we have used BPO obtained as a gift from the National Cancer Institute, the compound can be synthesized relatively easily from BP (3).

Due to the extreme sensitivity the method is especially useful for application in extrahepatic tissues and cultured cells. Since human hair follicles have been introduced as indicator organs for assessment of individual differences in susceptibility to chemical carcinogenesis, possibly reflected by differences in activities of carcinogen metabolizing enzymes among which GSH-T (7-9,16), we have studied the applicability of the present assay for human hair follicles. It was found that the enzyme could be detected in only one freshly isolated hair follicle. Thus, the present method is particularly useful for screening of high risk populations to identify interindividual differences in carcinogen metabolism.

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8

Rapid High-Performance Liquid Chromatographic Method for Detection of Interindividual Differences in Carcinogen Metabolism

ABSTRACT

A method is described for the analysis of the profile of organic solvent-soluble metabolites of benzo(a)pyrene formed by freshly isolated human hair follicles or cultured hair follicle keratinocytes. Both tissues produce qualitatively the same spectrum of metabolites with the 7,8- and 9,10-dihydrodiols representing the most important ones. Interindividual variation in dihydrodiol formation is much smaller than the range in phenol formation as analyzed from freshly isolated hair follicles of four different persons. Interindividual variation in total benzo(a)pyrene metabolism to organic solvent-soluble metabolites was about threefold. The method presented gives the opportunity of analyzing differences in the profile of various BP-metabolites and variations in the rate of BP-metabolism in an easily available human biopsy tissue, of epithelial origin. Moreover, the application of the methodology on cultured hair follicle keratinocytes can reveal the inducibility of various BP-metabolites after pre-exposure to polycyclic aromatic hydrocarbons.

INTRODUCTION

Interindividual variation in metabolism of carcinogens (mostly polycyclic aromatic hydrocarbons (PAH)) has been studied using various human tissues and cells (2,3,5,7,16,17,19,25,28). The observed variation seems to be primarily under genetic control (22). Differences in metabolic capacity to activate environmental carcinogens may result in differences in susceptibility to these

carcinogens. However, all the studies indicated above have used biopsy material that is not convenient for screening of populations because of practical reasons (e.g. with bronchus) or because of considerable different metabolic capacities compared to tissues susceptible to chemical carcinogenesis (e.g. in case of peripheral lymphocytes).

Human hair follicles have been suggested as a convenient biopsy tissue for screening individual differences in carcinogen metabolism since:

1. They are of epithelial origin, which is important in view of the fact that 90% of human cancers arises in epithelial cells (i.e. are carcinomas).
2. They are available from a large number of volunteers without any risk.
3. They have been shown able to activate and metabolize benzo(a)-pyrene (BP), a widely distributed carcinogen in our environment and a possible health hazard to humans (26).
4. Metabolism of BP to dihydrodiol derivates, the direct precursors of the ultimate carcinogens, the diol-epoxides, has been shown to be genetically determined for a large part in hair follicles (12; see also chapter 11).
5. Human hair follicle keratinocytes can be brought in culture (27), which enables the study of the effect of inducers and inhibitors of carcinogen-metabolizing enzymes on the metabolite pattern of carcinogens (13; see also chapter 13).
6. The response of BP metabolism in cultured hair follicle keratinocytes towards pre-exposure to PAH is comparable to that in cultured epithelial cells of the human bronchus, the target tissue for PAH-induced neoplasia (see chapter 10).

In this chapter the high-performance liquid chromatography (HPLC) analysis of the whole spectrum of organic solvent-soluble metabolites of BP in freshly isolated hair follicles and in cultured hair follicle keratinocytes is described. This methodology gives the opportunity to detect individual differences in carcinogen metabolism, using an epithelial biopsy material.

MATERIALS AND METHODS

Chemicals

[G-^{3}H]BP was purchased from the Radiochemical Centre (Amersham, Great Britain). BP was from Aldrich (Beerse, Belgium). NADPH was obtained from Boehringer (Mannheim, FRG) and gentamycin sulfate from Schering (Kenilworth, UK). Fetal calf serum, Minimal Essential Medium (with Earle's salts, MEM) and glutamine were purchased from Gibco (Glasgow, UK). Hydrocortisone was from Sigma (St. Louis, MO, USA), insulin from Organon (Oss, The Netherlands) and epidermal growth factor from Collaborative Research (Waltham, MA, USA). LiChrosorbRP-18 was obtained from Merck (Darmstadt, FRG). Synthetic BP standards were kindly provided by the NCI Chemical Repository at the IIT Research Institute (Chicago, IL, USA). Aquasol was obtained from New England Nuclear (Boston, MA, USA).

Collection of tissue and cell culture

Human hair follicles were obtained from the scalp of healthy volunteers using a pair of tweezers. Only hair follicles with visible bulb and sheath were used. Human hair follicle keratinocytes were cultured as described earlier (27) using a natural basement membrane-like extracellular matrix as growth substrate (bovine eye lens capsules) as described (9; see also chapter 2). Lens capsules and culture dishes (Epicult) were obtained from Sanbio B.V. (Nistelrode, The Netherlands). In short, hair follicles were placed on the lens capsules in the Epicult dishes and one drop of medium (MEM containing 15% fetal calf serum, 0.4 µg/ml hydrocortisone, 4 µg/ml bovine insulin and 10 ng/ml epidermal growth factor) was added. The cultures were placed in a humidified atmosphere of 5% carbon dioxide in 95% air. After three days when initial outgrowth started to appear, 0.3 ml of fresh medium was added. From then on the medium was changed twice a week. After 2-3 weeks the cultures had grown to confluency (about 2.10^5 cells/dish) and experiments were started.

Equipment

Throughout the study the following equipment was used: a liquid chromatograph (Waters Assoc., Milford, MA, USA) equipped with a U6K universal injector, two pumps (model 6000A), a solvent programmer (model 660), a UV-visible variable-wavelength detector (model 450), a reversed phase LiChrosorb RP-18 (5 µm) column (120 x 4.6 mm) and an Omniscribe recorder (Houston Instruments, Houston, TX, USA). Fractions were obtained with a programmable fraction collector FRAC$_{300}$ (Pharmacia, Uppsala,Sweden) and the radioactivity in the samples was analyzed with an LKB$_{1215}$ Rackbeta liquid scintillation counter (LKB, Stockholm, Sweden).

Analysis of [^{3}H]BP metabolism

[^{3}H]BP (5 µCi/ml culture medium) was purified by thin-layer chromatography (26), dissolved in ethanol, diluted with unlabeled BP and added to the cultures in a final concentration of 0.5 µM. In the case of freshly isolated hair follicles, incubation was performed with 60 hair follicles in 1 ml of 50 mM Tris-HCl, pH 8.5, containing 0.1 M sucrose, 3 mM $MgCl_2$, 10 µg/ml gentamycin sulfate, 2 mM NADPH and 0.5 µM [^{3}H]BP (5 µCi). After the incubation period (1 h for freshly isolated hair follicles and 24 h for the cultures) cells or hair follicles and medium were extracted three times with an equal volume of ethylacetate. For this purpose cultured cells were scraped in the medium with a bent Pasteur pipette and transferred to an Eppendorf tube. The collected organic phases were evaporated to dryness under a nitrogen stream, dissolved

in 50 µl of methanol and loaded on the HPLC column. From the time of injection the column was eluted with a gradient of 65-100% methanol in water. The gradient change was completed in 20 min. The constant flow rate was 0.8 ml/min. Eighty fractions of 0.4 min each (0.32 ml) were collected in minivials. As a consequence the last 30 fractions were obtained with the elution gradient in the end condition (100% methanol). The radioactivity in each fraction was determined with Aquasol as counting medium. After the collection of fractions background radioactivity could be washed out of the column within 10 min, and the column could be equilibrated for the next chromatographic analysis. A mixture of synthetic BP derivatives was used for the determination of the retention times of the various metabolites. For this purpose detection was carried out by UV spectroscopy at 254 nm. Identification of the [^{3}H]BP metabolites was achieved by comparison with the position of the authentic standards.

DNA assay

DNA in cultured hair follicle cells and freshly isolated hair follicles was determined by the mithramycin technique. After the metabolite extraction the tubes were centrifuged and the medium changed for distilled water. Then the cells or hair follicles were treated with pronase and the DNA measured as described earlier (10; see also chapter 3). Metabolite formation was expressed as fmol metabolite/µg DNA/h.

RESULTS AND DISCUSSION

Fig. 1 represents the separation of a mixture of BP and synthetic BP derivatives under the conditions described. The fraction numbers and the corresponding retention times of these BP metabolites are listed in Table 1. Fig. 2 shows the HPLC profile of organic solvent-soluble [^{3}H]BP metabolites after incubation of 60 freshly isolated hair follicles for 1 h (A) and of cultured hair follicle keratinocytes incubated for 24 h (B). In addition to diols, quinones and phenols, two early eluting components can be identified, especially in the cultured cells. The first one is a polar compound which hardly has any retention delay and which possibly is BP-3-yl hydrogen sulfate, an ethylacetate extractable sulfate conjugate. This metabolite has been identified in, for example, human and rodent lung cultures (4). However, certain tetrols can also elute in this region. The second early eluting component (fraction number 10-13) has been reported frequently in various tissues (19,20,23) and probably is one or more tetrol derivatives of BP. However, since absolute proof of identity of this component is not available, we have denoted this peak 'pre-9,10-diol'. It should be noted that tetrols and triols are evidence for the for-

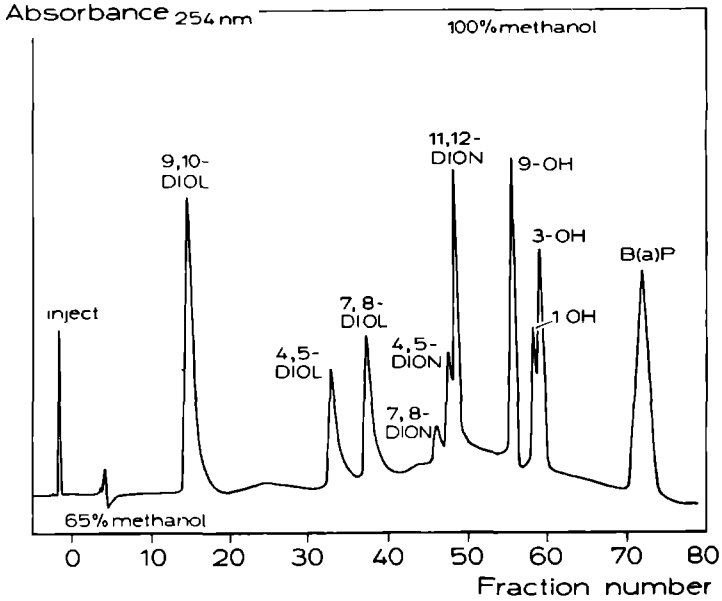

Fig. 1 Separation of a mixture of BP and synthetic BP derivatives under the conditions described in Materials and Methods. Abbreviations: diol = trans-dihydroxy-BP; OH = hydroxy-BP; dion = quinone-BP.

Table 1 Peak fraction numbers and corresponding retention times of various reference BP metabolites in the HPLC methodology described

compound*	peak fraction number	corresponding retention time (min)
9,10-diol	16	6.4
4,5-diol	33	13.2
7,8-diol	38	14.8
7,8-dion	46	18.8
4,5-dion	48	19.2
11,12-dion	49	19.6
9-OH	57	22.4
1-OH	59	23.6
3-OH	60	24.0
BP	72	28.8

*Abbreviations as in Fig. 1.

mation of diol-epoxides, the proposed ultimate carcinogens of PAH (6).

Although several quinone and phenol isomers of BP can be separated under the conditions described (Fig. 1), it is difficult to identify each peak in the quinone and phenol region exactly as one specific isomer. Therefore, all the metabolites which elute between fractions 44 and 52 have been taken together as quinones and the peaks between fractions 53 and 62 as phenols. The HPLC methodology described results in good separation of all the three dihydrodiol metabolites which have been isolated and characterized from various sources (6) as the (-)trans-4,5-dihydrodiol, (-)trans

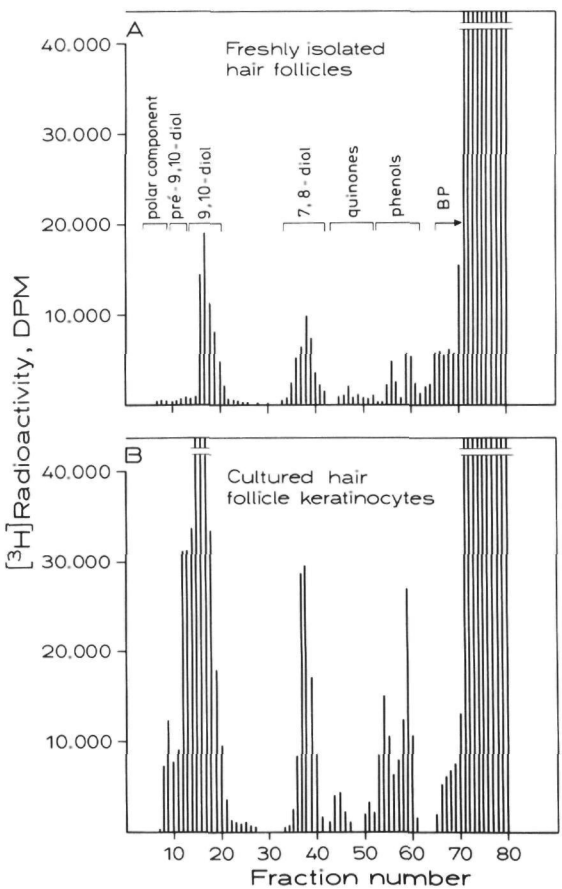

Fig. 2

HPLC profile of organic solvent-soluble [^{3}H]BP metabolites of freshly isolated hair follicles (incubated for 1 h) (A) and cultured hair follicle keratinocytes (incubated for 24 h) (B). Fractions 4-9 contain a polar component, possibly BP-4-yl hydrogen sulfate. Fractions 10-13 probably represent one or more tetrol metabolites of BP, called 'pre-9,10-diol'. The trans-9,10- and 7,8-dihydrodiol derivatives of BP elute between fractions 14 and 20, and 34 and 42, resp. Quinones appear between fractions 44 and 52, and phenols between fractions 53 and 62. Unmetabolized [^{3}H]BP elutes from fraction 65.

-7,8-dihydrodiol and the (-)trans-9,10-dihydrodiol. Fig. 2A shows that in freshly isolated hair follicles more than 70% of the organic solvent-soluble metabolites are represented by the 7,8- and 9,10-dihydrodiol metabolites. In contrast, freshly isolated hair follicles hardly metabolize BP to the 4,5-dihydrodiol derivative. Two other important groups of organic solvent-soluble BP metabolites formed by freshly isolated hair follicles are represented by quinones and phenols although they are formed to a lesser extent than the dihydrodiols.

We have analyzed BP metabolism in freshly isolated hair follicles from a number of volunteers. The range of formation of each of the metabolite groups is illustrated in Table 2. It is obvious that the variation in dihydrodiol formation is much smaller than the range in variation in phenol formation. Since dihydrodiols result from epoxide hydrolase activity and phenols reflect aryl hydrocarbon hydroxylase activity these findings suggest that the interindividual variation in epoxide hydrolase is lower than that for aryl hydrocarbon hydroxylase. In fact, low interindividual variation for epoxide hydrolase and large interindividual differences in aryl hydrocarbon hydroxylase activity have been reported

Table 2 Range of formation of various [^{3}H]BP metabolites and groups of metabolites in freshly isolated hair follicles of a number of volunteers

compound	range of variation*	mean ± S.D.
polar component	0.6- 1.5	1.1 ± 0.5
pre-9,10-diol	1.3- 2.6	1.8 ± 0.7
9,10-diol	41.1-46.3	44.4 ± 2.6
7,8-diol	29.1-31.5	29.9 ± 1.4
quinones	3.6- 8.5	5.3 ± 2.7
phenols	10.7-23.1	17.5 ± 6.3

**expressed as the percentage of the total amount of organic solvent-soluble metabolites.*

n = 4. Abbreviations as in Fig. 1. For further explanation see text.

Table 3 Formation of [^{3}H]BP metabolites or metabolite groups in freshly isolated human hair follicles and cultured hair follicle keratinocytes from the same donor

compound	freshly isolated hair follicles	cultured hair follicle keratinocytes
polar component	4 ± 1	22 ± 5
pre-9,10-diol	4 ± 1	30 ± 5
9,10-diol	130 ± 20	96 ± 16
7,8-diol	92 ± 17	33 ± 6
quinones	13 ± 3	10 ± 3
phenols	73 ± 27	31 ± 12
total	316 ± 43	222 ± 35

Abbreviations as in Fig. 1. The data represent the mean values for three persons and are expressed as fmol per µg DNA per h.

for various human tissues (see, for example, ref. 1 and references therein) including human hair follicles (16; see also chapter 12). Interindividual variation in total BP metabolism to organic solvent-soluble metabolites was about three-fold as analyzed in the present study.

The metabolite pattern of cultured human hair follicle keratinocytes is qualitatively comparable with freshly isolated hair follicles with dihydrodiols representing the major metabolite group, phenols and quinones the minor ones. Due to the longer incubation time the total level of metabolism is higher than in freshly isolated hair follicles (Table 3). However, the mean rate of BP metabolism in cultured cells is somewhat lower than in freshly isolated hair follicles. This can be ascribed to the greater formation of dihydrodiols, quinones and phenols in freshly isolated hair follicles. In contrast, the amount of the more polar organic solvent-soluble metabolites is greater in cultured hair follicle keratinocytes than in freshly isolated hair follicles. The observation that at longer incubation times there is about the same amount of 9,10-dihydrodiol and much more tetrols indicates that 9,10-dihydrodiol is an end-point in BP metabolism and 7,8-dihydro-

diol can be further metabolized in the diol-epoxides.

Cultured hair follicle keratinocytes offer the opportunity to investigate BP metabolism after induction of the enzyme aryl hydrocarbon hydroxylase. High levels of induced activity of this enzyme have been correlated with genetic susceptibility to PAH-induced neoplasia in some studies (8,17), while other authors failed to confirm this (21,24). One of the sources of controversy can be the choice of human biopsy tissue, peripheral lymphocytes. In view of the prevalence of carcinomas, the use of human keratinocytes seems to be a more appropriate alternative. It has already been shown that the response of BP metabolism towards pre-exposure to benz(a)anthracene in cultured hair follicle keratinocytes, is qualitatively comparable with that in cultured human bronchial epithelial cells, the target tissue of PAH-induced neoplasia. In contrast, murine epidermal cells, a frequently studied cell type in chemical carcinogenesis, do respond differently to pre-exposure to benz(a)anthracene as compared with human epidermal keratinocytes (13; see also chapter 13).

The method presented here offers the advantage of rapid analysis of BP metabolism in an easily available biopsy tissue of epithelial origin, the human hair follicle. Both differences in the profile of the various BP metabolites and variations in the rate of BP metabolism can be monitored easily. The application of the methodology on cultured hair follicle cells gives the opportunity to measure inducibility of BP metabolites after pre-exposure to PAH. Together with the recently developed assays for carcinogen-metabolizing enzymes in hair follicles (11,15), the method may contribute to identification of high-risk populations (see also chapter 5-7).

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Covalent Binding of Benzo(a)pyrene Metabolites to DNA of Cultured Human Hair Follicle Keratinocytes

ABSTRACT

Primary cultures of human hair follicle keratinocytes were established by using a basement membrane-like growth substrate, the bovine eye lens capsule. A method was adapted for the isolation of [^{3}H]benzo(a)pyrene (BP)-modified DNA from the cellular outgrowth of only one hair follicle (approximately 2 x 10^5 cells). In a routine procedure hair follicle keratinocytes were incubated with 0.5 µM [^{3}H]BP during 24 h. The purified DNA was subjected to enzymic hydrolysis and the adducts were analyzed by Sephadex LH-20 column chromatography followed by HPLC. Only one major adduct, which represented 60-80% of the total radioactivity, could be identified. This adduct co-chromatographed with the marker adduct resulting from the trans-addition of the N-2-amino group of guanine to the 10-position of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene.

Co-incubation with 7,8-benzoflavone (0.3 µM), an inhibitor of cytochrome P-448, and with 1,1,1-trichloropropene-2,3-oxide (0.2 µM), an inhibitor of epoxide hydrolase, resulted in a marked inhibitory effect (15% of the control binding) and a large increase (300% of the control value) in BP-DNA binding respectively. Induction of aryl hydrocarbon hydroxylase activity in the cultures with 5,6-benzoflavone (10 µM) or benz(a)anthracene (10 µM) caused a decrease (46 and 75% of the control value respectively) in BP-DNA binding. Since the ratio between 'total' binding (as calculated from the specific activity of the isolated DNA) and 'true' binding (represented by the BP-nucleoside adducts eluted from the HPLC-column) appeared to be rather constant, 'total' binding may serve as a good representative of 'true' binding. Using cultures from

hair follicles of eight different persons, interindividual variation was 4-fold, with a mean binding level of 2.4 x 10^9 molecules BP/µg DNA. Since it has been demonstrated that the formation of dihydrodiols of BP - among which the proximate carcinogen 7,8-dihydrodiolBP - in freshly isolated hair follicles is genetically determined for a large part (Hukkelhoven et al., Clin. Genet., 21: 53, 1982), the interindividual differences in binding can possibly reflect individual variation in susceptibility to BP-induced neoplasia.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are metabolized to a wide range of organic solvent-soluble and water-soluble metabolites and it is known that this metabolic activation is necessary for their observed adverse biological effects (9,13,32). Benzo(a)pyrene (BP) has been used as a prototype compound for investigations concerning biological fate and action of PAH. In order to better understand the mechanisms by which PAH exert their mutagenic, cell-transforming and tumorigenic or carcinogenic effects in man, it has become apparent that the following considerations should be taken into account:

1 Chemical carcinogens, among which PAH, can enter the human body through surface epithelia and the majority of their carcinogenic actions is exerted in these tissues. Therefore it is important to study metabolism in human surface epithelia.

2 Intact cell systems containing a full complement of both oxidative and conjugative enzymes should be utilized, since it has been demonstrated that these better reflect the *in vivo* situation (7,12).

3 In order to study the wide interindividual differences in susceptibility to carcinogens an easily obtainable human biopsy tissue has to be used. Since it has been argued that prevention of chemically induced cancer, especially bronchus carcinoma, should have priority in the control of the disease (15), such a biopsy tissue would facilitate this strategy by the possible identification of high risk groups.

We have proposed that human hair follicles are a biopsy tissue which might be very useful in the evaluation of the effects of biochemical parameters in carcinogen metabolism on individual

susceptibility to these substances (23,47). Recently, a method for the routine culture of primary hair follicle keratinocytes has been described (49). BP-metabolism of freshly isolated hair follicles as well as cultured hair follicle keratinocytes has been studied in detail (24; see also chapter 8) and compared to that of the target-tissue of PAH-induced neoplasia, the tracheobronchial epithelium (25; see also chapter 10).

Several studies have indicated that 7,8-dihydrodiolBP and the corresponding 7,8-dihydrodiol-9,10-epoxide respectively are the proximate and ultimate carcinogenic metabolites of BP, which are also responsible for the quantitatively most important covalent binding to DNA (10,41). Administration of BP in *in vivo* animal studies or to cells *in vitro* results in the formation of BP-DNA adducts of which the major one is generated by 9/10 trans-addition of the 2-amino group of guanine to the C-10 position of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE I) (28,30,31). The formation of this adduct has been shown to correlate with mutagenesis in mammalian cells (36) and carcinogenesis in mouse skin (35). Moreover, a clear correlation has been found between the carcinogenic effect of a series of PAH on mouse skin and the level of the respective hydrocarbon-DNA adducts in that tissue (40). However, using mouse strains with different susceptibility no correlation could be observed between the levels of binding and the reported susceptibility of these strains (39). Therefore the exact relation between DNA-modification by chemical carcinogens and biological activity of these substances is still unclear. Since the distribution of BP-DNA adducts is specific and reproducible for each species (11) and attention should be given to a target cell-type, we have described in this chapter the formation and identification of BP-DNA adducts in cultured human hair follicle keratinocytes. Moreover, the kinetics of the binding as well as the effects of modulators of carcinogen metabolizing enzymes (aryl hydrocarbon hydroxylase (AHH) and epoxide hydrolase (EH)) on the binding process are discussed.

MATERIALS AND METHODS

Chemicals

[G-^{3}H]BP (specific activity 40-70 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, UK. Aquasol was from New England Nuclear, Boston (Mass.). 4,6-diamidino-2-phenylindole.2HCl (DAPI) and 5,6-benzoflavone (5,6-BF) were obtained from Serva, Heidelberg, FRG. 7,8-benzoflavone (7,8-BF), benz(a)anthracene (BA) and 1,1,1-trichloropropene-2,3-oxide (TCPO) were from Aldrich, Beerse,Belgium. Alkaline fosfatase, calf spleen fosfodiesterase and bovine pancreas RNase were from Boehringer Mannheim, Mannheim, FRG. Proteinase K and Lichrosorb RP18 were purchased from Merck, Darmstadt, FRG. Calf thymus DNA,phenol, bovine pancreas DNase 1 and 8-hydroxy quinoline were from Sigma, St. Louis. Sephadex LH-20 was obtained from Pharmacia, Uppsala, Sweden. Reference BP-metabolites (4,5-epoxyBP, BPDE I and (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE II)) were donated from the National Cancer Institute Chemical Repository (IIT Research Institute, Chicago Ill.).

Cell culture

Human hair follicle keratinocytes were cultured as described earlier (49) in an especially developed culture dish, the Epicult (21; see also chapter 2). A basement membrane-like growth substrate (bovine eye lens capsules) was used. Epicults and bovine eye lens capsules were provided by Sanbio B.V., Nistelrode, The Netherlands. Confluency of the primary cultures in the Epicult was reached after about three weeks.

Alkylation of DNA

The following procedure was developed to isolate DNA from the outgrowth of one single hair follicle covering the eye lens capsule. [^{3}H]BP was purified chromatographically before each experiment (46) and finally dissolved in ethanol. It was added to the cultures at a standard concentration of 0.5 µM. The ethanol concentration in the medium was 1%. After the required incubation period (standard 24 h) the medium was removed from the Epicult and the cultures shortly washed with 0.2% trypsine and 0.5% EDTA in Tyrode buffer (0.8% NaCl, 0.02% KCl, 0.005% $NaH_2PO_4.H_2O$, 0.1% $NaHCO_3$, 0.2% glucose, adjusted to pH 7.4). Then the cells were treated with this solution for 20-30 min. The harvested cells (about 2.10^5 cells/Epicult) were transferred to a tube and centrifuged (5 min, 1000 g) in the presence of a few drops of calf serum. The collected cells were washed three times with Tyrode buffer. The final pellet was lysed during 10 min at ambient temperature and 5 min at 37°C with 200 µl 10 mM Tris-1 mM EDTA, pH 7.5 (TE) to which NaCl (final concentration 0.2 M) and SDS (final concentration 0.5%) were added. Protein was hydrolyzed by addition of proteinase K at a final concentration of 200 µg/ml and incubation during 2 h at 37°C. The resulting mixture was extracted with PCI-solution (phenol : chloroform : isoamylalcohol, 25 : 24 : 1, 0.5% hydroxyquinoline and saturated with TE) according to the scheme illustrated in Fig. 1. The combined water phases were extracted with one volume of chloroform to remove residual phenol. The water phase was collected and adjusted to 0.2 M NaCl, after which 2.5 volumes ethanol were added. The precipitated DNA was dissolved in 100 µl TE and treated with RNase (final concentration 100 µg/ml) which had been heat-treated (20 min, 80°C) to remove residual DNase activity. SDS was added to a final concentration of 0.5% after which the proteinase K treatment and the DNA extraction scheme was repeated. The isolated DNA was dissolved in 1 ml water and the purity assessed spectrophotometrically (A260/A280>1.85).

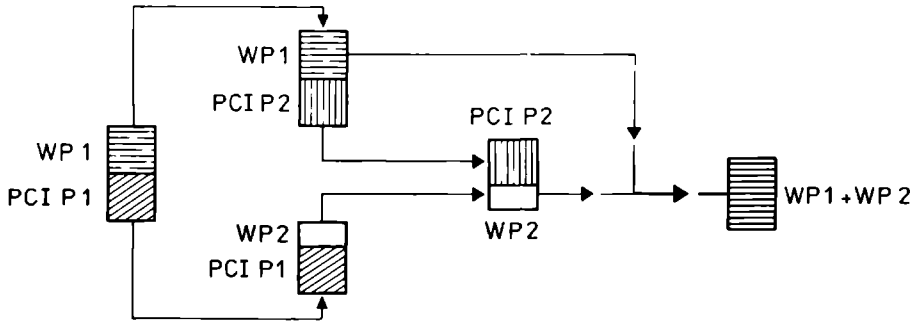

Fig. 1 Extraction scheme for the isolation of DNA from cultured hair follicle keratinocytes. PCI P is the organic phase (phenol : chloroform : isoamylalcohol, 25 : 24 : 1, 0.5% hydroxy quinoline and saturated with 10 mM Tris, 1 mM EDTA, pH 7.5). WP represents the water phase (10 mM Tris, 1 mM EDTA, pH 7.5). Note that PCI P1 is re-extracted with 1/3 volume of buffer (WP 2).

Quantification of DNA-binding

A sample (50 µl) of the dissolved DNA was added to 10 ml Aquasol and the radioactivity measured in a LKB Rackbeta liquid scintillation counter. The quantity of DNA present in a sample was determined by the DAPI-method described earlier (22; see also chapter 3). The binding of BP to DNA, expressed as molecules BP/µg DNA, was calculated from the specific activity of BP, the radioactivity of the DNA sample and the quantity of DNA present.

Enzymic degradation and column chromatography of DNA

DNA samples were enzymatically degraded to deoxyribonucleosides by dissolving the precipitated DNA in 0.01 M Tris, 0.01 M $MgCl_2$ (pH 7.0) and adding 260 Kunitz U. DNase I. After 4-6 h incubation period (37°C), 1 ml Tris (0.1 M, pH 9.0) was added to adjust the pH to 9.0. Then 0.1 U phosphodiesterase is added and the mixture incubated for another 44-50 h, after which 2.5 U alkaline fosfatase is added (incubation time 24-48 h, 37°C). The hydrolyzed DNA can be lyophilized prior to column chromatography. For this purpose the samples were fractionated by elution from a 80-cm column of Sephadex LH-20 with a gradient from 30 to 100% methanol. The DNA sample is loaded on the column dissolved in 30% methanol. The flow rate is 0.25 ml/min. Fractions of 3 ml are collected and 300 µl samples were assayed for radioactivity using 5 ml Aquasol. Fractions that elute after the initial oligonucleosides (i.e. after 200 ml of the gradient) were pooled and lyophilized prior to HPLC-analysis.

HPLC-analysis of BP-DNA adducts

Characterisation of the BP-nucleoside products was performed by HPLC using the methodology of Weinstein et al. (48). The column was developed with a 25-80% concave methanol-water gradient at 50°C at 0.9 ml/min flow rate. 75 µl Aceton was added to 1 liter 25% methanol to reduce the UV base-line shift. 1.13 ml Fractions were collected and mixed with 5 ml Aquasol for analysis of radioactivity. UV detectable markers were synthesized as described earlier (28) and included reaction products of 4,5-epoxyBP, BPDE I and BPDE II with calf thymus DNA.

Effects of incubation time and BP-concentration on the DNA-binding

The cultures of human epidermal keratinocytes consisted of tightly packed polygonal cells with a characteristic epithelial appearance (Fig. 2). Sometimes filamentous structures, possibly keratin filaments, could be seen in the cytoplasm. Detailed electron microscopic analysis has been presented elsewhere (50). Since cytotoxic effects have been described for cultured human keratinocytes above 1 µM BP (20) and already above 0.15 µM for human mammary epithelial cells (42), we have chosen a BP concentration of 0.5 µM for routine experiments.

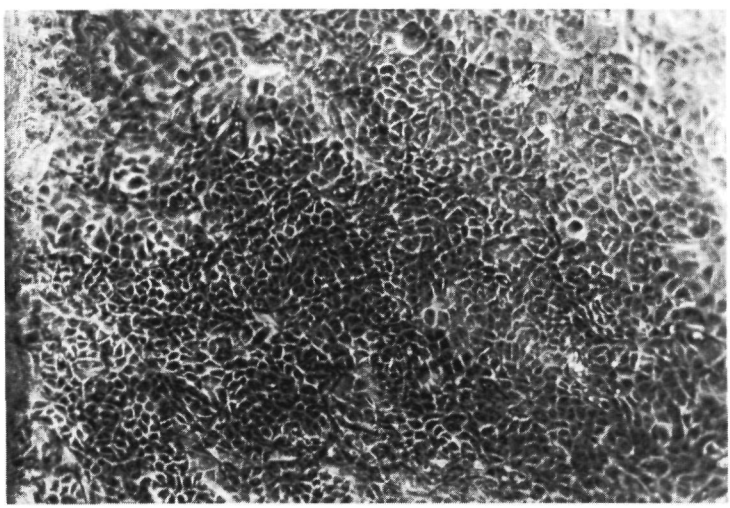

Fig. 2 Phase contrast microscopical picture of primary human hair follicle keratinocytes after 3 weeks in culture.

During the first 4 h of incubation the increase in DNA binding is maximal. Maximal binding levels are reached after 24 h (Fig. 3A). Fig. 3B shows that at 1 µM BP, DNA binding levels after 24 h incubation differ about 2-fold compared to those at a BP concentration of 0.5 µM (for this experiment a culture of a different volunteer, exhibiting lower binding levels, than in Fig. 3A has been used).

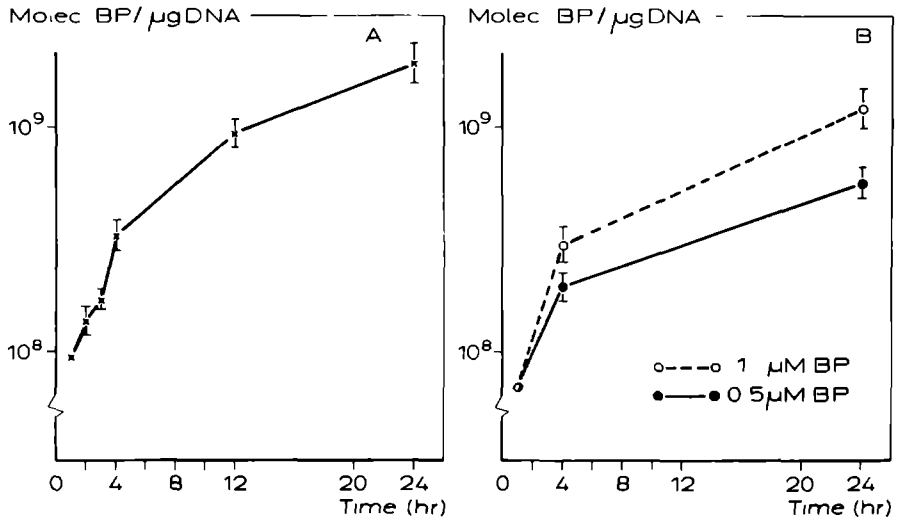

Fig. 3 (A) Time course of 'total' binding of BP to the DNA of cultured hair follicle keratinocytes. Cultures were incubated in triplicate for each time-point with 0.5 µM.
(B) 'Total' binding of BP to the DNA of cultured hair follicle keratinocytes at 0.5 µM BP (•—•) and 1.0 µM BP (o---o). For this experiment cultures of a different individual than in Fig. 3A were used.

Removal of BP-DNA adducts

After a 24 h incubation with [^{3}H]BP the medium was removed and the cells rinsed with fresh medium without BP. Then incubation was continued for 24 h. After the 24 h post BP-treatment, DNA was isolated and BP-DNA binding assessed in 3 dishes. Only 8% of the adducts were shown to be removed in this period.

Effect of modulation of activities of carcinogen metabolizing enzymes on BP-DNA binding

The effects of inhibitors and inducers of carcinogen metabolizing enzymes were tested on cultures of human hair follicle keratinocytes. 7,8-BF (an inhibitor of cytochrome P-448 and thus of AHH) and TCPO (an inhibitor of EH) were co-incubated with [^{3}H]BP during 24 h at a concentration of 0.3 µM and 0.2 µM resp. A marked inhibitory effect on BP-DNA binding was observed with 7,8-BF resulting in about 15% of the control binding activity.

Table 1 Influence on BP-DNA binding

treatment	BP-DNA binding	% of control
none	1.5×10^9	100
7,8-benzoflavone	2.3×10^8	15
1,1,1-trichloro-propene oxide	4.5×10^9	300
none	4.3×10^9	100
5,6-benzoflavone	3.2×10^9	75
benz(a)anthracene	2.0×10^9	46

Confluent cultures of hair follicle keratinocytes treated with [^{3}H]BP (0.5 µM) during 24 h were co-incubated with 7,8-benzoflavone (0.3 µM) and 1,1,1-trichloropropene oxide (0.2 µM). For the analysis of the influence of AHH-inducers, cultures were pre-treated with 5,6-benzoflavone (10 µM) or benz(a)anthracene (10 µM) during 24 h. After thoroughly rinsing they were incubated with [^{3}H]BP (0.5 µM) during 24 h. Binding values reflect 'total' binding as calculated from the specific activity of the isolated DNA and are expressed as molecules BP bound/µg DNA. Each point represents the mean of cultures of two individuals with three dishes per individual (except for the benz(a)anthracene-treated cultures where three individuals were tested).

The addition of TCPO resulted in a large increase in DNA binding (300% of the control value; Table 1). The effects of induction of AHH by BA and 5,6-BF were studied by addition of BA (10 µM) dissolved in DMSO (final concentration 0.5%) or 5,6-BF (10 µM) dissolved in ethanol (final concentration 0.5%) to the cultures, 24 h before addition of [^{3}H]BP. Before [^{3}H]BP was added, the cultures were 5 times rinsed with 0.9% NaCl solution to avoid possible competition effects between 5,6-BF or BA and BP. Addition of both inducers resulted in a decrease in DNA binding. The mean DNA binding levels for the BA-treated cultures (3 different persons and 3 dishes per culture) was 46% of the control value and for the 5,6-BF-treated cultures (2 different persons and 3 dishes per culture) 75% of the control value (Table 1).

Interindividual variation in BP-DNA binding

From 8 different persons DNA binding levels were assessed in duplicate. The mean binding was 2.4 x 10^9 molecules BP/µg DNA. The variation was about 4-fold with a lowest value of 1.1 x 10^9 and a highest value of 4.6 x 10^9 molecules BP/µg DNA.

Sephadex LH-20 column chromatography

The Sephadex LH-20 chromatography elution profile of the BP-nucleic acid adducts present in the hydrolysate of DNA isolated from [^{3}H]BP treated hair follicle keratinocytes, is shown in Fig. 4. In all the cultures analyzed, radioactivity was confined to three main regions of the chromatogram. Firstly, two early eluting components after about 30 ml and 60 ml, respectively and a region of radioactivity between 210-250 ml elution volume. Since with the chromatographic system employed nucleic acid adducts are known to elute in the nonpolar regions of the elution gradient, only the products eluted after 200 ml are considered as modified deoxyribonucleosides. The early eluting material appears to be

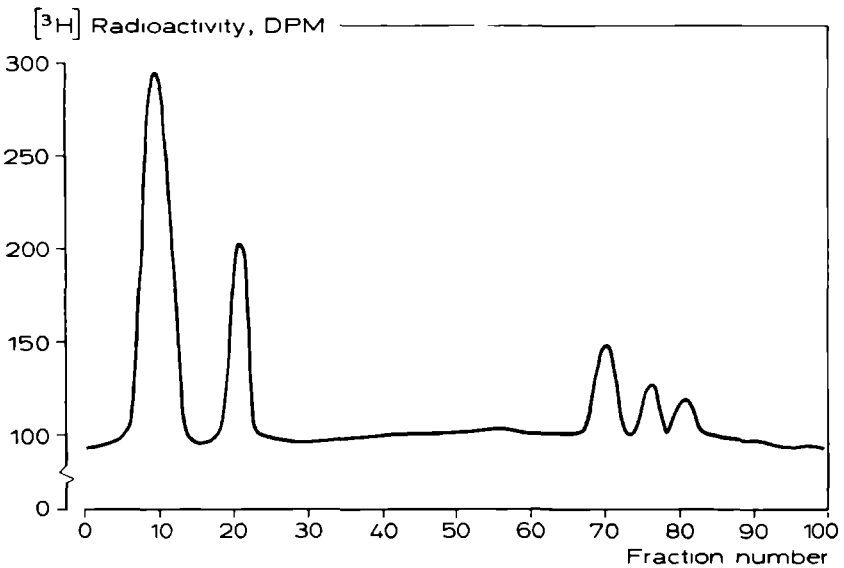

Fig. 4 Elution profile from Sephadex LH-20 column of hydrolysates, prepared as described in the text, of DNA isolated from cultured human hair follicle keratinocytes incubated with [^{3}H]BP (0.5 µM).

due to tritium exchange into normal (unmodified) deoxyribonucleosides and to resistance of some of the hydrocarbon-DNA adducts to complete enzymic hydrolysis (39).

HPLC analysis of DNA carcinogen adducts

After Sephadex LH-20 chromatography, the adducts between BP and the deoxyribonucleosides of cultured hair follicle keratinocytes, were analyzed by HPLC and identified by using well characterized UV-markers as reference compounds (28). Only 1 major adduct could be identified unequivocally and represented 60-80% of the total radioactivity. This adduct is formed by reaction of the exocyclic 2-aminogroup of guanine with the C-10 position of BPDE I. The remaining radioactivity did not clearly co-chromatograph with any of the other synthesized markers (Fig. 5).

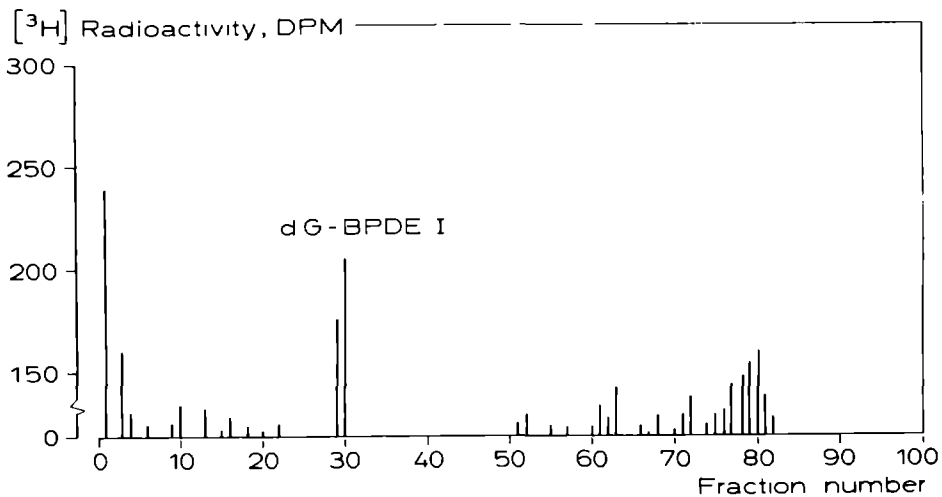

Fig. 5 HPLC profile of DNA adducts formed by human hair follicle keratinocytes incubated with [^{3}H]BP (0.5 µM). Fractions 29 and 30 co-chromatograph with the synthesized BPDE-dG adduct.

Since BP is a ubiquitous pollutant linked to human cancer (16, 26), studies of its metabolic fate in human model systems are important for understanding the chemical carcinogenesis of BP. The recent advances in culturing hair follicle keratinocytes provide the opportunity to study this process in a human cell-type highly relevant for chemical carcinogenesis and easily accessible to permit population studies for the identification of high risk groups.

The metabolism of BP by cultured human hair follicle keratinocytes has been studied in detail (24) and was shown to be qualitatively similar to that in cultured human bronchial epithelial cells, the target cell-type for PAH-induced neoplasia (25; see also chapters 8 and 10). There is compelling evidence that some of these metabolites - but also those of many other carcinogens - react with cellular DNA. If DNA replication proceeds on such a modified template before altered bases or nucleotides are excised by enzymatic repair processes, DNA damage may be genetically fixed. Thus, the extent of carcinogen-induced (promutagenic) DNA damage and the capacity of cells to repair such damage, represent critical events in the initiation of carcinogenesis.

In the present investigation DNA-BP interactions have been studied in cultured hair follicle keratinocytes. It has been described that AHH-inducers (such as 5,6-BF, TCDD, Aroclor 1254) strongly inhibit BPDE-DNA adduct formation *in vivo* in different organs in mice (for a summary of these data see 2). This is consistent with our results of cultured hair follicle keratinocytes where we observed a decrease in DNA binding as a result of pretreatment of the cultures with 5,6-BF or BA. However, these *in vitro* results contrast markedly with findings obtained in other *in vitro* systems such as microsomes, isolated perfused organs, tissue slices and hepatocytes (for a summary see 52). Other data on the effects of inducers of AHH on BP-DNA binding in keratinocytes are not available in the literature. The exact relevance of our finding is, however, unclear. It cannot be excluded that the decreased binding is merely a result of 'dilution' of the BP-metabolites over the increased pool of mRNA and protein as

caused by the induction process.

7,8-BF, which *in vitro* inhibits mono-oxygenase activity associated with cytochrome P-448, results in a marked decrease in BP-DNA binding in cultured hair follicle keratinocytes. This is consistent with other studies (8 and references therein) which report decreased *in vitro* binding of various types of BP-metabolites to nucleic acids in the presence of 7,8-BF. We have shown previously that BP-metabolism in freshly isolated hair follicles and hair follicle keratinocytes in culture can be almost completely abolished by addition of 7,8-BF, suggesting that a large part of the mono-oxygenase system in hair follicle cells is associated with cytochrome P-448 (46). Since it has been described that 7,8-BF reduces the binding of BP to DNA in rat liver nuclei from 3-MC treated animals but not from control or phenobarbital-treated rats (1), the finding in this study of reduced BP-DNA binding upon 7,8-BF incubation further supports the idea that constitutive mono-oxygenase levels in hair follicle cells are predominantly cytochrome P-448 associated. This would also explain the predominance of the BPDE I adducts, which are thought to be preferentially produced by the action of cytochrome P-448.

Co-incubation of the hair follicle keratinocytes with the EH-inhibitor TCPO resulted in an increase in adduct formation. However, the exact biological importance of this phenomenon is unclear, since it has been shown that the increase in binding did not correspond to products other than unidentified material (1). It has been suggested that the increase in binding by inhibition of EH is caused by inhibition of conversion of non-K-region epoxides to the corresponding phenols, by blocking the metabolism of 4,5-epoxyBP to 4,5-diolBP and by enhancing the quinone-oxide pathway by which quinones are further metabolized resulting in intermediates which can interact with DNA (8). However, it has to be realized that TCPO can also have an effect on AHH-activity (53) so that the increase in DNA binding by TCPO can partly be mediated by modification of AHH-activity.

Grunberger (19) has recently reported that in contrast to BP, N-hydroxy-N-2-acetylaminofluorene, the proximate carcinogenic form of the hepatocarcinogen 2-acetylaminofluorene, does not bind covalently to the DNA of cultured human epidermal keratinocytes.

This can be interpreted as evidence that metabolism of carcinogens and covalent binding to DNA in cultured human keratinocytes correlates well with the cell specificity exhibited by chemical carcinogens.

The low repair after 24 h post BP-treatment (8%) indicates the relative resistance of the adducts in hair follicle keratinocytes. This is consistent with cultured human mammary epithelial cells where a decrease of about 20% was noted after 72 h post BP-treatment (42).

The only adduct which could be identified in the cultured hair follicle keratinocytes was that which arises from interaction of BPDE I at the C-10 position of BP and the exocyclic amino group at the N-2 position of guanine. The predominance of the BPDE I-dG adduct has also been demonstrated in cultured human epidermal keratinocytes (43). The adduct has also been detected in relatively large amounts in various other human cell systems such as bronchial explants (27), colon explants (5), lung cells (14) and human mammary cells (34,42). It is important to note that the nature of the BP-nucleoside adducts is strongly dependent on the type of test-system used. When BP is incubated with DNA and mouse or rat liver microsomes, at least nine distinct BP-metabolite-nucleoside complexes can be identified after hydrolysis of DNA, with a 9-hydroxy-4,5-oxide-nucleoside adduct being the most important (38, 44,45). When isolated perfused liver is incubated with BP four peaks can be separated, the most abundant being the nucleoside adduct of BPDE I (29). BP-modified DNA from skin epidermis, trachea, bronchi, isolated hepatocytes or cells in culture almost always contains only one major peak, that of BPDE I associated with guanine (3,4,27,30,33,42,51). Therefore the integrity of cells is crucial for the nature of the adducts.

In four different cultures from which we analyzed the isolated DNA by HPLC, the ratio of the specific binding (represented by the metabolite-nucleoside adducts beyond fraction 20) to total binding (calculated from the radioactivity in the isolated DNA) was fairly constant. Although it has been noted that 'true' binding instead of 'total' binding yields better correlations among DNA binding, mutagenesis and carcinogenesis, the experimental settings for DNA binding experiments in our cultures are so repro-

ducible that 'total' binding may serve as a good representative of 'true' binding. The use of 'total' binding as a biochemical parameter in carcinogen metabolism has the advantage over 'true' binding that it does not necessitate the use of rather time-consuming DNA hydrolysis and subsequent HPLC-analysis, which is especially important in routine analysis.

Using cultures of eight different persons, we have found about 4-fold differences in total BP-DNA binding. Theall et al. (43) reported a 2-3-fold difference using five different cultures of human epidermal keratinocytes. The same order of variability was reported by Parkinson and Newbold (37) using six serially cultivated strains of human epidermal keratinocytes grown on 3T3 feeder layers. These rather small interindividual differences contrast with the very wide quantitative differences in binding values obtained with human tissues in organ culture. For cultured bronchus, esophagus and colon interindividual variation was reported to be 75-, 99-, and 100-fold respectively (17,18,6). These differences between cultured cells and organ culture can partly be explained by the relative cellular homogeneity - in contrast to organ culture - and by the rather long culture time of keratinocytes (2-3 weeks) which ensures that environmental, pharmacological, nutritional and hormonal influences impinging on the cells when they are in the donor are minimized. Therefore, the interindividual differences obtained with hair follicle keratinocytes can reflect the genetically determined balance between toxification and detoxification of BP-metabolites, and thus the individual susceptibility to BP-induced neoplasia. Using twin analysis we have demonstrated earlier that the formation of dihydrodiols of BP - among which the proximate carcinogen 7,8-dihydrodiolBP - in freshly isolated hair follicles is for a large part genetically determined (23).

The ability to measure the formation of DNA-carcinogen adducts in cultured keratinocytes from an easily available biopsy tissue, the hair follicle, will further facilitate investigations concerning the relation between this biochemical parameter and the carcinogenic potential of the chemical in a given individual.

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PART 3

HUMAN HAIR FOLLICLES AS INDICATORS FOR INDIVIDUAL DIFFERENCES IN CARCINOGEN METABOLISM

10

Human Hair Follicles and Cultured Hair Follicle Keratinocytes as Indicators for Individual Differences in Carcinogen Metabolism

ABSTRACT

Benzo(a)pyrene (BP)-metabolism in freshly isolated human hair follicles, cultured hair follicle keratinocytes and cells cultured from human bronchial epithelium was analyzed by high performance liquid chromatography. All three types of tissues resulted in quantitatively comparable amounts of the most important organic solvent-soluble metabolites: 9,10-dihydrodiol-BP, 7,8-dihydrodiol-BP, quinones, and phenols. Besides these metabolites two early eluting compounds were detected: one possibly is BP-3-yl-hydrogen sulfate, the other probably consists of one or more tetrols. Aryl hydrocarbon hydroxylase in both cell-types was highly inducible after pre-exposure to benz(a)anthracene. Water-soluble metabolites were quantitatively unimportant in both types of cultured cells and appeared to be primarily glucuronide and sulfate conjugates with the monohydroxides and the 7,8-dihydrodiol of BP. This metabolic pattern is compared to that of monocytes and lymphocytes which have been used frequently in population studies and with data from other types of human epithelial cells. It is concluded that human hair follicles and cultured keratinocytes from these organs are useful for detection of individual differences in carcinogen metabolism.

INTRODUCTION

Differences in the biological response to carcinogens, especially polycyclic aromatic hydrocarbons (PAH), have been described among species, individuals, tissues, and cell-types (2,9,12,21).

The existence of interspecies variation precludes direct extrapolation of data in experimental animals to the human situation. Therefore the use of human biopsy tissue (e.g., bronchus, esophagus, colon, liver etc.) is important in studies involved in chemical carcinogenesis. However, these tissues are not suited for population studies to investigate the interindividual variation in susceptibility to carcinogens. Since the regulation of certain carcinogen-metabolizing enzymes seems to be primarily under genetic control (22,23) it should be possible to detect high risk populations.

Although interindividual variation can result from every step in chemical carcinogenesis, e.g., metabolic activation of carcinogens, binding to DNA, repair of damaged DNA and promotion of initiated cells, most efforts have been spent to correlate differences in carcinogen metabolism to susceptibility to carcinogens. Lymphocytes have been used frequently for this purpose but unfortunately the results have been ambiguous, some studies correlating aryl hydrocarbon hydroxylase (AHH)-inducibility to the risk of developing bronchogenic carcinoma (8,18), others not able to confirm this (1,25). Probably, lymphocytes are an inconvenient cell-type to predict an individual's overall ability to activate PAH. The reason for this might be the different metabolic capacity of these cells compared to the target cell-type for PAH-induced neoplasia, the tracheo-bronchial epithelium.

For reasons described in chapter 8 human hair follicles have been suggested as a convenient biopsy-tissue for identifying individuals with increased risk for developing chemically induced cancer (see also ref. 5,11,13-16,27-29).

Normal skin biopsies are less suited for use in population studies since they often cause scars and since pure keratinocytes cannot be obtained from them in primary culture. Moreover, hair follicles are located fairly deep in the skin and are thus less vulnerable to possible enzyme inducers contacting with skin. Furthermore, skin biopsies are often contaminated with dermal layers which exhibit a quantitatively different carcinogen metabolism (26).

In this chapter the metabolic pattern of BP in freshly isolated hair follicles and cultured hair follicle keratinocytes is analyzed

and compared to the target cell-type for PAH-induced carcinogenesis, human bronchial epithelial cells.

MATERIALS AND METHODS

Chemicals

[G-^{3}H]-BP (specific activity 40 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, U.K. and was purified chromatographically before each experiment. Unlabeled BP was obtained from Aldrich, Beerse, Belgium. NADPH was obtained from Boehringer Mannheim and gentamycine sulfate from Schering, Kenilworth, U.K. Fetal calf serum, Minimal Essential Medium (with Earle's salts, MEM) and glutamine were purchased from Gibco, Glasgow, U.K. β-Glucuronidase (Type 1) and arylsulfatase were purchased from Sigma, St. Louis, USA. Lichrosorb RP_{18} was obtained from Merck, Darmstadt, FRG. Synthetic BP-derivatives were kindly provided by the National Cancer Institute Chemical Repository at the IIT Research Institute, Chicago, USA. Aquasol and EN^{3}HANCE which was used as an autoradiography enhancer were obtained from New England Nuclear, Boston, USA. For autoradiography Kodak XAR-51 films were used.

Collection of tissue

Human hair follicles were obtained from the scalp of healthy volunteers using a pair of tweezers. Volunteers using tar shampoo were excluded from the study. Only hair follicles with visible bulb and sheath were used. Human bronchial tissue was obtained at bronchoscopy and transported to the laboratory in ice-cold MEM supplemented with 10% fetal calf serum and 10 μg/ml gentamycine sulfate. All bronchial biopsies were taken for diagnostic purposes. Only macroscopically unaffected tissue was used.

Cell cultures

Human keratinocytes originating from hair follicles were cultured as described earlier (29) using a natural basement membrane-like extracellular matrix (bovine eye lens capsules) as growth substrate (10, see also chapter 2). Lens capsules and culture dishes (Epicults) were obtained from Sanbio B.V., Nistelrode, The Netherlands. Human bronchial epithelial cells were cultured as described by Hukkelhoven et al. (14; see also chapter 14). Only primary cultures were used in the experiments.

Equipment

Throughout the study the following equipment was used: a liquid chromatograph (Waters ass., Milford, USA) equipped with a U6K 'universal injector', two pumps (model 6000A), a solvent programmer (model 660), a UV-visible variable wavelength detector (model 450), a reverse-phase Lichrosorb 5-μm column (120 x 4.6 mm) and an Omniscribe recorder (Houston Instruments, USA). Fractions were obtained with a 'programmable fraction collector' $FRAC_{3000}$ (Pharmacia, Uppsala, Sweden) and the radioactivity in the samples was analysed with an LKB_{1215} Rackbeta liquid scintillation counter.

Analysis of BP-metabolism (organic solvent-soluble metabolites)

HPLC analysis of BP-metabolites from both cell culture systems and from freshly isolated hair follicles was performed as described in chapter 8. A mixture of synthetic BP-derivatives was used for the determination of the retention

time of the various metabolites. For this purpose detection was carried out by UV spectroscopy at 254 nm. For further identification of the crucial dihydrodiols, reference compounds were also subjected to t.l.c. (solvent system toluene/ethanol (9 : 1) and the R_f-values compared with those of the metabolites from the HPLC-analysis.

Water-soluble metabolites

For analysis of conjugate formation, the media were re-extracted three times with two volumes of ethylacetate to remove any residual BP or organic solvent-soluble BP-metabolites. Then the media were incubated with β-glucuronidase (1 mg/ml) and arylsulfatase (300 µg/ml) for 3 h at 37°C. After incubation the media were extracted three times with ethylacetate, the extract was evaporated and the residue applied to silica gel t.l.c. using a solvent mixture of toluene/ethanol (9 : 1). After spraying with autoradiography enhancer radioactive spots were examined by exposure of the t.l.c. plates to X-ray film.

Induction of aryl hydrocarbon hydroxylase activity in cultures

Aryl hydrocarbon hydroxylase (AHH) was induced in cultures of hair follicle keratinocytes and bronchial epithelial cells by exposure to culture medium containing 10 µM benz(a)anthracene (BA) in dimethylsulfoxide (DMSO) as described earlier (12; see also chapter 13). After a 16 h exposure-time, the cultures were rinsed four times with 0.9% NaCl-solution, and AHH-activity was measured according to the fluorometric determination of phenolic BP-metabolites (15; see also chapter 5). For quantitation of AHH-activity, DNA was measured in the cultures according to the mithramycin method described in chapter 3. AHH-activity was expressed as pmol phenolic BP-metabolites/µg DNA/h. The inducibility ratio was calculated by dividing induced AHH-activity by constitutive AHH-levels.

RESULTS

Bronchial epithelial cell cultures from four patients were subjected to HPLC after incubation with ^{3}H-BP. From four other persons both freshly isolated hair follicles and cultured hair follicle keratinocytes were analyzed. Fig. 1 shows a typical example of the HPLC-profile of freshly isolated hair follicles, hair follicle keratinocytes in culture and cultured bronchial epithelial cells. All three types of tissue result in almost the whole series of organic solvent-soluble BP-metabolites: 9,10- and 7,8-dihydrodiols, quinones, and several monohydroxy BP derivatives. In addition to these metabolites two early-eluting components can be observed: one polar component which elutes between 1.5 and 3.5 min (fraction 4-9) and which possibly is BP-3-yl-hydrogen sulfate. This metabolite has been identified in e.g., human and rodent lung cultures (6). For further identification of this early-eluting component we have isolated the compound from a t.l.c. plate

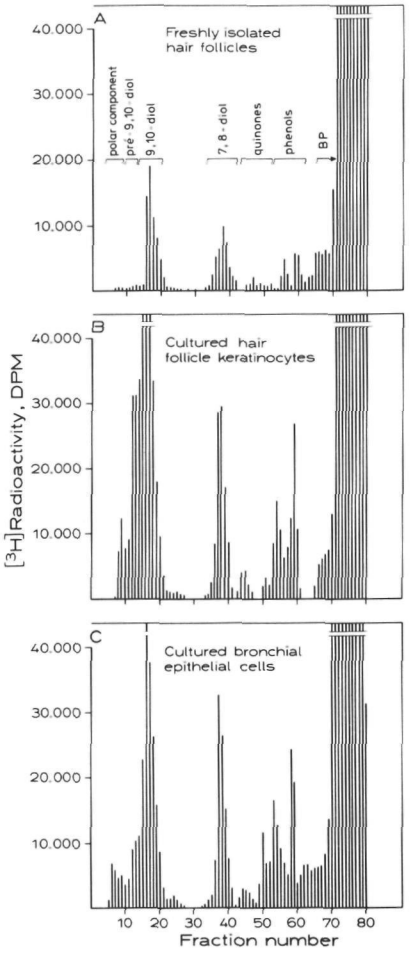

Fig. 1

HPLC separation of ^{3}H-BP derivatives obtained from freshly isolated hair follicles (A), cultured hair follicle keratinocytes (B) and cultured human bronchial epithelial cells (C). Fractions 4-9 contain a polar component, possibly BP-3-yl-hydrogen sulfate. Fractions 10-13 probably represent one or more tetrol metabolites of BP and is called 'pré-9,10-diol'. The trans-9,10- and 7,8-dihydrodiol derivatives of BP elute between fractions 14-20 and 34-42 resp. Quinones appear between fraction 44 and 52 and phenols between fraction 53 and 62. Unmetabolized BP elutes from fraction 65. Freshly isolated hair follicles were incubated during 1 h, the cultures during 24 h.

with 95% ethanol and recorded the fluorescence emission and excitation spectra of it. They were found largely identical with the synthetic BP-3-yl-hydrogen sulfate as described by Cohen et al. (6). The other polar component has a retention time of about 4.5 min (fraction 10-13) and probably can be attributed to one or more tetrols. However, since absolute proof on the identity of this compound is missing, it has been designated as 'pré-9,10-diol-BP' because it is the compound which elutes just before the 9,10-dihydrodiol-BP. It is obvious that dihydrodiols represent the most important metabolites of BP in all three systems. However, 4,5-

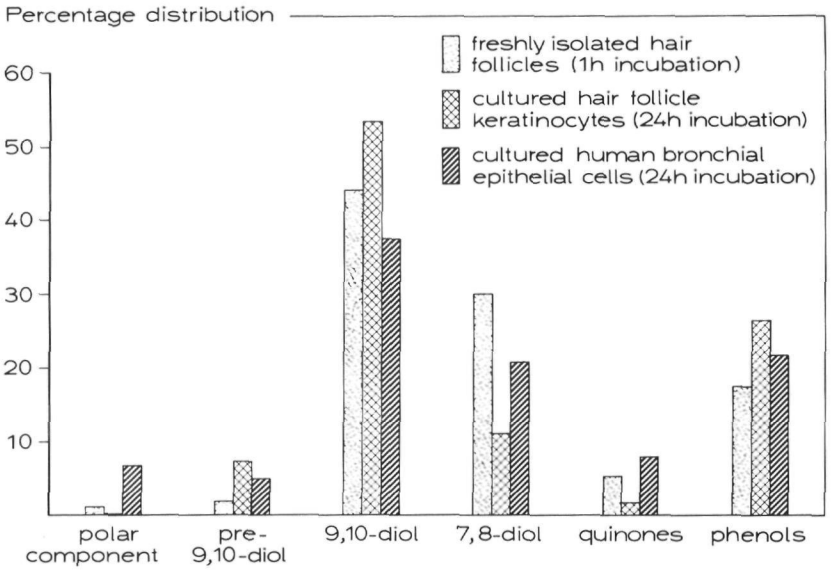

Fig. 2 Percentage distribution of ^{3}H-BP metabolites or metabolite groups in freshly isolated hair follicles, cultured hair follicle keratinocytes and cultured human bronchial epithelial cells. Data represent the mean of four analyses of each tissue type obtained from different persons. Freshly isolated hair follicles and cultured hair follicle keratinocytes were obtained from the same volunteers.

dihydrodiol-BP is formed in negligible amounts. Phenols and quinones are also formed but in smaller amounts than the dihydrodiols. Although several monohydroxy derivatives of BP can be separated, identification of each peak in this region is difficult to achieve due to possible overlap with other phenols. The same holds true for the quinones. Therefore all quinone and phenol-derivatives have been taken together. Both boiled hair follicles (10 min) and hair follicles incubated at 0°C did not show any metabolite formation.

Fig. 2 represents the percentage distribution of organic solvent-soluble BP-metabolites after incubation of freshly isolated hair follicles, cultured hair follicle keratinocytes, and cultured human bronchial epithelial cells. It is obvious that all the three systems do form the same range of organic solvent-soluble metabolites and that the relative distribution of each metabolite group

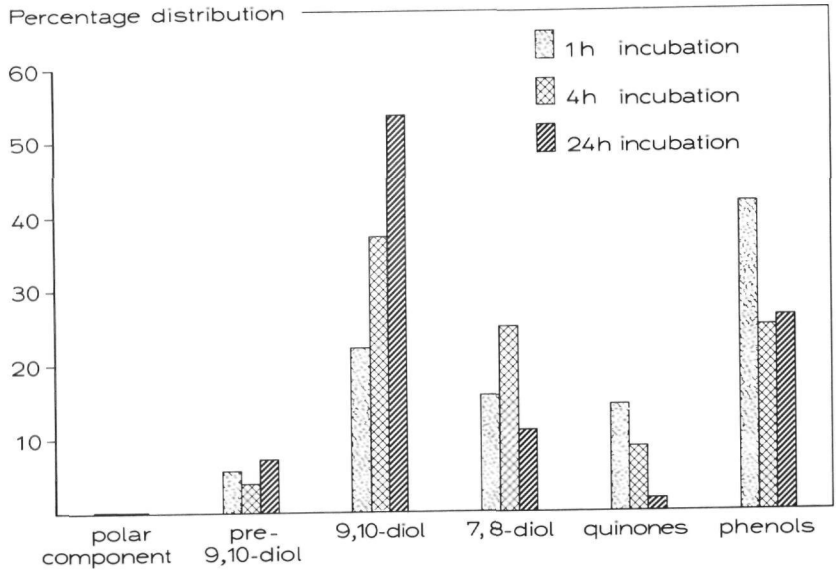

Fig. 3 Percentage distribution of ^{3}H-BP metabolite(groups)in cultured hair follicle keratinocytes after different incubation times. All cultures were obtained from the same volunteer.

is comparable. From the analysis of BP-metabolism in hair follicles and cultured hair follicle keratinocytes from different individuals it can be concluded that the interindividual differences in dihydrodiol formation are less than those in phenol formation (data not shown). This conclusion is in agreement with earlier observations using larger groups of volunteers (17; see also chapter 12).

Fig. 3 shows the change in percentage distribution of organic solvent-soluble BP-metabolites from the same donor as a function of the incubation time for cultured hair follicle keratinocytes. The amount of 9,10-dihydrodiol-BP does increase with incubation time which suggests that this metabolite can be an endpoint in BP-metabolism. Quinones have decreased to very low levels in the 24-h incubation. The level of phenols declines from a 1-h to a 4-h incubation but stabilizes at longer incubation times. The decrease in quinone formation reveals that in the experimental procedure described spontaneous oxidation of the labile 1-OH and 3-OH phenols to quinones does not occur.

Small amounts of phenols and 7,8-dihydrodiols are released when the medium of the cultured cells is treated with β-glucuronidase and arylsulfatase. However, these glucuronide and sulfate conjugates represented less than 3% of the total amount of organic solvent-soluble metabolites. In freshly isolated hair follicles no conjugate formation could be detected probably due to the short incubation time of 1 h. The results suggest that human hair follicle keratinocytes and bronchial epithelial cells have low UDP-glucuronyl transferase and sulfate transferase activities. Since even after exhaustive ethylacetate extraction and arylsulfatase/β-glucuronidase treatment some radioactivity still remains in the water-phase and because of the existence of relatively high levels of glutathione transferase activity in hair follicles (see chapter 7) we suppose that most of the radioactivity left results from ^{3}H-BP-glutathione conjugates. However, on the basis of control experiments using medium without cells, it can be concluded that some radioactivity left results from non-specifically bound ^{3}H-BP to serum proteins.

AHH-activity in both cell types is higly inducible after pre-exposure to BA. The range in inducibility ratios for cultured keratinocytes (n=9) was between 2.0 and 8.4 (mean 4.4 ± 1.8) and for bronchial cells (n=4) between 2.7 and 5.1 (mean 3.9 ± 1.1).

DISCUSSION

Individual differences in carcinogen metabolism may lead to different susceptibility to the biological effects of these compounds. In order to measure BP-metabolism in man an easily obtainable source of human tissue is required. Up till now lymphocytes and monocytes and to a lesser extent pulmonary macrophages have been used for this purpose. Using cultures of blood lymphocytes and monocytes it has been shown that the interindividual variation in AHH-activity and inducibility is largely genetically determined. However, whether an individual's AHH-activity or inducibility in blood cells can be related to his susceptibility to PAH carcinogenesis is still the subject of controversy today (7).

The unability to relate carcinogen metabolism to carcinogen susceptibility can possibly be ascribed to the different metabolic capacities and characteristics between the more accessible cells and the target cells for chemical carcinogenesis. The need to select biopsy material and model systems for PAH-carcinogenesis in man on the basis of comparable metabolite patterns has been argued (2,14; see also chapter 14).

Human hair follicles have been suggested as an alternative for blood lymphocytes and monocytes for various reasons. In the present study we have compared BP-metabolism in freshly isolated hair follicles and cultured hair follicle keratinocytes with the target cell-type for PAH-induced carcinogenesis, the human bronchial epithelium. In all three systems dihydrodiols represent more than 60% of the total amount of organic solvent-soluble BP-metabolites while quinones and phenols are less important metabolite groups (about 7 and 20% respectively). The metabolism of BP in cultured blood lymphocytes and monocytes has been studied in detail by HPLC-analysis (24). From this study it appears that dihydrodiols comprise only about 15% of total organic solvent-soluble metabolites in monocytes and about 10% in lymphocytes. Total phenol synthesis is about 75% in lymphocytes as well as monocytes. Another striking difference is the ratio between 7,8- and 9,10-dihydrodiol-BP. In all three systems in our study the amount of 9,10-dihydrodiol-BP is about two times that of 7,8-dihydrodiol-BP and comprises 35-50% of all organic solvent-soluble BP-metabolites. In contrast, in both lymphocytes and monocytes the amount of 9,10-dihydrodiol-BP is very low (about 1%) and the amount of 7,8-dihydrodiol-BP relatively high.

Our results with cultured bronchial epithelial cells are in accordance with those obtained using cultured human bronchus tissue (3). In this study the same percentage distribution of organic solvent-soluble metabolites as in our system was found. Moreover, the metabolite pattern of our cultured human keratinocytes and bronchial epithelial cells is comparable with that in human mammary epithelial cells (4) and with cultured human epidermal cells (20). Strikingly, in all human epithelial cell systems mentioned before, 7,8-dihydrodiol-BP is formed in smaller amounts

than the 9,10-dihydrodiol-BP. Moreover, in these cultures, but not in lymphocytes and monocytes, metabolites in the tetrol region could be detected. These results indicate that epithelial cells, including cultured hair follicle keratinocytes, convert 7,8-dihydrodiol-BP to tetrols, presumably with the ultimate carcinogen, the 7,8-diol-9,10-epoxide of BP, as an intermediate. The apparent low activity of UDP-glucuronyl transferase and sulfate transferase in both cultured hair follicle keratinocytes and bronchial epithelial cells is in agreement with the very low activity of these conjugating enzymes present in human epidermal keratinocytes as reported by Kuroki et al. (19). The observed greater interindividual variation in phenol formation than in dihydrodiol formation has been confirmed earlier by analyzing these metabolite groups separately (17; see also chapter 12) and can reflect larger interindividual differences in AHH-activity than in epoxide hydrolase activity.

Both cultured hair follicle keratinocytes and bronchial epithelial cells are inducible with respect to the formation of phenolic BP-metabolites after pre-exposure to BA. However, it is shown elsewhere in this thesis (chapters 13 and 14) that the formation of dihydrodiol metabolites in both cell-types is not affected by this treatment in contrast to e.g. murine skin and bronchial cells (12,14). Moreover, based on the (limited) number of experiments performed, it can be concluded that the range of interindividual differences in induction ratios in cultured human hair follicle cells and bronchial epithelial cells is quantitatively comparable.

In conclusion, BP-metabolism in freshly isolated hair follicles and in cultured hair follicle keratinocytes is comparable with cultured bronchial epithelial cells, the target cell-type for PAH-induced carcinogenesis and with human epidermal and mammary epithelial cells, two other cell-types for which evidence of a role of chemical carcinogens in the development of skin and breast cancer respectively has been provided. In contrast, very large differences do exist between these cells and cultured lymphocytes and monocytes. Cultured primary hair follicle keratinocytes offer the additional advantages of relative cellular homogeneity - in contrast to human epithelial organ culture where large sample

variations and mixtures of cell-types are present - and the absence of the selective pressure of repeated passage in culture that is characteristic of 'immortal' epithelial human cell lines. On the other hand the culture time of hair follicle keratinocytes (2-3 weeks) seems sufficiently long to minimize environmental, pharmacological, nutritional, and hormonal influences impinging on the cells when in the donor. In view of the results obtained, human hair follicles and cultured hair follicle keratinocytes may be very useful to test the hypothesis that individuals differ in their metabolic capacity to activate environmental carcinogens and that this may result in individual differences in susceptibility to these substances.

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Genetic Factors in Benzo(a)pyrene Metabolism to Dihydrodiols by Human Hair Follicles

ABSTRACT

Basal levels of benzo(a)pyrene metabolism were measured in hair follicles of seven monozygotic twins, eight dizygotic twins and ten pairs of unrelated individuals. Organic soluble metabolites were separated by thin-layer chromatography, visualized by autoradiography and quantified by scanning of the films. Activity was expressed as pmol 7,8- and 9,10-dihydrodiol metabolites of benzo(a)pyrene per μg DNA per hour. Intra-twin differences in benzo(a)pyrene metabolism for monozygotic twins were smaller than for dizygotic twins and intra-pair differences for dizygotic twins were smaller than for pairs of unrelated individuals. The results clearly suggest that individual differences in benzo(a)pyrene metabolism towards dihydrodiols in hair follicles are partly genetically determined. Thus, hair follicles may be used for investigation on the suggested relation between genetic predisposition to carcinogen-induced cancer and individual differences in carcinogen metabolism. The relevance of these findings to research into the induction of neoplasms by carcinogenic substances in epithelial tissues is discussed.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are thought to exert their carcinogenic action after metabolism to a complex mixture of over 20 intermediary metabolites (3). A mixed function oxidase system located in the endoplasmic reticulum and in nuclei is a key factor in the metabolic activation of PAH to mutagenic and

carcinogenic derivatives.

The ability of tissues and cells to metabolize PAH has frequently been estimated by fluorometric measurement of the phenolic metabolites of benzo(a)pyrene, a ubiquitous representative of the PAH (9). This activity, commonly referred to as aryl hydrocarbon hydroxylase (AHH) can be elevated in certain cell cultures or animal tissues after pretreatment with benz(a)anthracene or various other xenobiotics (10). Much interest has been focused on the relationship between benzo(a)pyrene metabolism and the risk for human cancer, particularly bronchogenic carcinoma and laryngeal cancer. Studies with mitogen-stimulated lymphocytes have shown a trimodal distribution of the inducibility of AHH (7). In this study, patients with lung cancer predominantly fell in the class of high inducibility of AHH. However, other investigators failed to confirm this conclusion (12). Twin studies suggested that genetic factors accounted for much of the total interindividual variation in AHH inducibility using monocytes (11) and lymphocytes (1). Paigen et al. (13) concluded that basal and induced AHH-activity as well as inducibility are inherited traits. The failure to show a relationship between AHH-inducibility and the risk for human cancer may be caused by an inappropriate choice of the source of human tissue: lymphocytes. Lymphocytes are not common target-cells for chemical-induced carcinogenesis. Furthermore, AHH induction in lymphocytes requires the addition of mitogens, thus creating a nonphysiological condition.

Human hair follicles are an easily obtainable source of epithelium, the target cell-type for 80-90% of all human malignancies. The presence of AHH in hair follicles has been shown recently (14; see also chapter 5). In the present study, basal levels of benzo(a)pyrene metabolism were measured in hair follicles obtained from healthy monozygotic and dizygotic twins and from non-related individuals. Attention was focused on the formation of the 7,8- and 9,10-dihydrodiol metabolites of benzo(a)pyrene. These intermediates, especially the 7,8-dihydrodiol, are the direct precursors of the diol-epoxides which are thought to be the ultimate carcinogenic metabolites of benzo(a)pyrene (15).

MATERIALS AND METHODS

Collection of tissue

Freshly plucked hair follicles were obtained from the scalps of healthy Caucasian twins (seven monozygotic and eight dizygotic twins). Zygosity was determined by measurement of 10 different blood groups. Each pair of twins was of the same sex, except twin pair 10. As controls, pairs of unrelated individuals were used.

Measurement of benzo(a)pyrene metabolism

Benzo(a)pyrene metabolism for each person was determined in duplicate, with 30 hair follicles per incubation. Each pair was assayed 3-8 times. The procedure was identical to that described elsewhere (6; see also chapter 13). In short, after incubation of 30 hair follicles in 1 ml of a hypotonic Tris-buffered solution (pH 7.55) in the presence of NADPH (2 mmol), $MgCl_2$ (3 µmol) and [^{3}H]-benzo(a)pyrene (3 µCi; specific activity 19 Ci/mmol), organic soluble metabolites were extracted with ethylacetate. After drying of the organic phase and separation on t.l.c. plates benzo(a)pyrene metabolites were visualized by autoradiography. The films were scanned and, after comparison with known amounts of radiolabeled benzo(a)pyrene (BP), BP metabolism was expressed as pmol diols formed per µg DNA in 1 h. One unit of activity was defined as 0.1 pmol diols formed per µg DNA in 1 h.

DNA assay

DNA in the hair follicles was determined by the mithramycine test (4) with modifications according to Hukkelhoven et al. (5). After the extraction with ethylacetate, the aqueous phase was removed. Then 1 ml of distilled water and 10 µl of a pronase-solution (50 mg/ml, obtained from Calbiochem, Lucerne and free of nucleases) was added to the hair follicles. The follicles were incubated with the protease for 1 h. Then 50 µl of a mithramycin stock solution (Mithracin©, Pfizer Limited, New York, 200 µg mithramycine/ml in 300 mM $MgCl_2$) was added. After mixing, the samples were centrifuged and the fluorescence was measured using a spectrofluorometer (Perkin-Elmer 650-40) at an excitaion wavelength of 440 nm and an emission wavelength of 540 nm. Herringsperm DNA (Sigma, St. Louis) was used for a calibration curve. The treatment with distilled water leads to disruption of the hair follicle cells, after which pronase can digest the proteins of the liberated nucleoprotein complexes, making the DNA accessible to binding with mithramycin.

RESULTS

Fig. 1 shows a typical example of an autoradiograph obtained after incubation of hair follicles from a monozygotic and a dizygotic twin. After comparison with known reference BP derivatives (obtained by courtesy from the NCI Chemical Repository at the IIT Research Institute, Chicago, Ill.), the major metabolites can be identified as 7,8-dihydro-7,8-dihydroxyBP and 9,10-dihydro-9,10-dihydroxyBP. All pairs were assayed 3-8 times. Due to the instab-

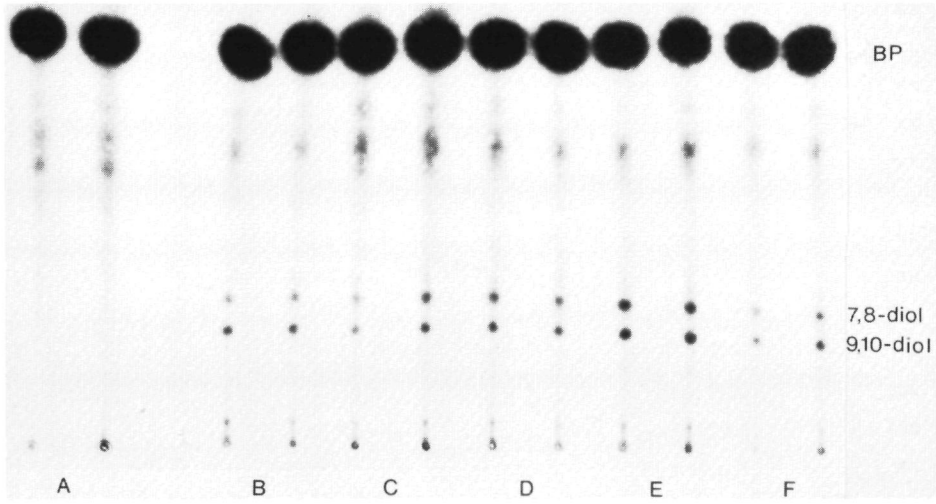

Fig. 1 Autoradiograph of a chromatogram of the ethylacetate soluble metabolites of [G-^{3}H]BP in hair follicles (determinations in duplicate): (A) blanks; (B) 30 hair follicles obtained from the author of this thesis; (C) and (D) a monozygotic twin; (E) and (F) a dizygotic twin. Abbreviations: BP = benzo(a)pyrene 7,8-diol = 7,8-dihydro-7,8-dihydroxyBP 9,10-diol = 9,10-dihydro-9,10-dihydroxyBP

ility of phenolic metabolites of BP on t.l.c., no attempt was made to quantitate the formation of these intermediates.

It was noted that the amounts of metabolite formed for the same individual assayed at different times could vary considerably. When assayed simultaneously, monozygotic twins showed more similar values than did dizygotic twins. In Fig. 2 the first two assays of each pair are shown. It is evident that the intra-twin differences for monozygotic twins are less than for dizygotic twins (mean intra-twin difference for monozygotic twins is 1.21 ± 1.05, and for dizygotic twins 2.43 ± 1.91), while dizygotic twins exhibit smaller differences than pairs of unrelated individuals (mean intra-pair difference is 2.98 ± 1.74). When we define an intra-pair difference in BP metabolism of less than 25% as 'insignificant', it is possible to score the percentage of monozygotic twins, dizygotic twins and pairs of unrelated individuals who have 'equal'

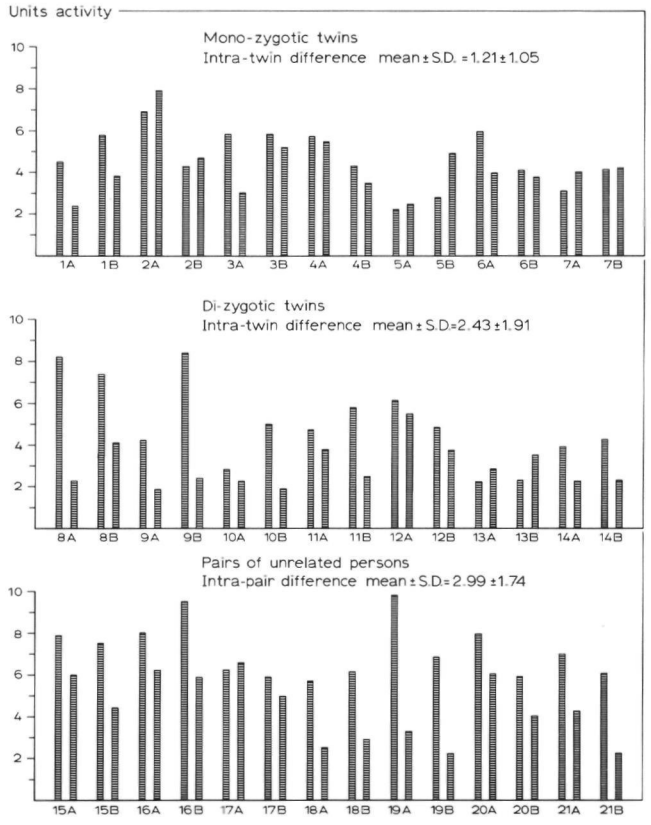

Fig. 2 Values of BP metabolism in hair follicles of seven monozygotic twins, seven dizygotic twins and seven pairs of unrelated individuals. The values shown are those obtained at first and second assay of each pair. Note the increasing intra-pair difference in the sequence: monozygotic twins, dizygotic twins, pairs of unrelated individuals.

BP metabolism. These figures are shown in Table 1. All determinations are included in this table (up to eight separate determinations for one pair). It is obvious that the percentage of pairs showing roughly equal BP metabolism decreases in the sequence: monozygotic twins, dizygotic twins and pairs of unrelated individuals.

Table 1 Genetic factors in benzo(a)pyrene metabolism in human hair follicles

	number of pairs	number of determinations	% with equal BP metabolism*
monozygotic twins	7	42	54
dizygotic twins	8	27	39
unrelated pairs	10	37	34

**Figures represent percentage of assays of monozygotic twins, dizygotic twins and pairs of unrelated individuals showing insignificant intra-pair differences in BP metabolism towards dihydrodiols (differences less than 25% of the highest value of a pair).*

DISCUSSION

The reported correlations between inducibility of AHH in various strains of mice and their susceptibility to carcinogenesis by some PAH (8), together with the finding that bronchogenic carcinoma is associated with high AHH-inducibility in lymphocytes (7), generated high expectations about our understanding of chemical-induced tumors. A subsequent study, however, failed to show such a relation (12). The conflicting nature of the results may be caused by several factors, such as isolation and culture of lymphocytes and treatment with mitogens. Moreover, the use of lymphocytes may be an inappropriate choice of tissue, because most human malignancies arise in epithelium-derived tissues. Finally, the measurement of a single type of metabolite, the BP-phenols, which do not bear any close relation to the ultimate carcinogen, the 7,8-diol-9,10-epoxide of BP (2), may not always be a sufficient reflection of total BP metabolism.

Using the epithelium-derived human hair follicle as biopsy tissue, we were able to show that the mean intra-pair difference in BP metabolism is smaller in monozygotic than in dizygotic twins and smaller in dizygotic twins than in pairs of unrelated individuals. Furthermore, the percentage of assays yielding insignificant intra-pair differences was greatest in monozygotic twins and smallest in pairs of unrelated individuals. These results clearly

suggest that genetic factors account for a large part of the total interindividual variation in basal levels of BP metabolism towards dihydrodiols.

We have shown elsewhere (6) that cultured keratinocytes originating from hair follicles do not show increased BP metabolism towards the crucial dihydrodiols after pre-exposure to benz(a)-anthracene. This is in contrast to several animal cell cultures. If *in vivo* tissue of epithelial origin were also not inducible with regard to PAH metabolism towards biologically active intermediates, basal levels of this metabolism might be the most important determinant of susceptibility to carcinogens. The evidence presented here that differences in BP metabolism of hair follicles are partly genetically determined offers the opportunity to investigate whether there is a correlation between levels of BP metabolism and susceptibility to carcinogen-induced neoplasm. The use of hair follicles for these assays therefore, may resolve the controversy in the literature about a possible relationship between AHH-activity and the risk of developing cancer on exposure to PAH.

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12

Formation of Phenolic and Dihydrodiol Metabolites of Benzo(a)pyrene in Freshly Isolated Human Hair Follicles Occurs Independently

ABSTRACT

Freshly isolated hair follicles of 20 adult non-smoking volunteers were assayed for formation of phenolic and dihydrodiol metabolites of benzo(a)pyrene (BP). In each of a total of 14 experiments two volunteers were assayed simultaneously, and the ratios of both phenolic and dihydrodiol metabolites of BP between the two individuals were determined. It was obvious that the mean interindividual variation in formation of phenolic metabolites was greater than the variation in formation of dihydrodiol metabolites. No correlation existed between the amount of both types of metabolites formed. These observations indicate that for detection of differences in carcinogen metabolism to assess individual susceptibility to the carcinogenic action of polycyclic aromatic hydrocarbons, measurements of phenolic and dihydrodiol metabolites of BP are not interchangeable.

INTRODUCTION

Carcinogen metabolizing enzymes may determine susceptibility to chemical carcinogens, especially polycyclic aromatic hydrocarbons (PAH) (12,18). Thus, measurement of these enzymes in an appropriate human biopsy tissue might lead to identification of high-risk populations. However, it is not yet known what the enzymic determinants of the balance between carcinogen activation and deactivation are, and which of them are related to cancer risk. On the one hand, aryl hydrocarbon hydroxylase (AHH) is the first enzyme involved in the formation of active metabolites (epoxides), on the

other hand epoxide hydrolase (EH) provides the substrates for the formation of the supposed ultimate carcinogens of PAH, the diol-epoxides. Furthermore, conjugating enzymes may contribute to detoxification of carcinogens. Human hair follicles have been introduced as a convenient biopsy tissue for studies on individual differences in carcinogen metabolism (6-8). In this study we have analyzed the formation of phenolic and dihydrodiol metabolites of benzo(a)pyrene (BP) in human hair follicles, to investigate whether a correlation between both metabolic routes of BP exists. Phenolic and dihydrodiol metabolites of BP do reflect the activity of AHH and EH, respectively, in the cell.

METHODS

Volunteers

Volunteers were non-smoking male and female adults. From a list of 20 volunteers, pairs were selected at random. A total of 14 experiments were performed. As a consequence 8 persons were assayed twice. Hair follicles were collected from the scalp at random. Only hair follicles with visible bulb and sheath were used.

Measurement of BP-metabolism towards phenols and dihydrodiols

Phenolic metabolites of BP, which result from AHH-activity, were determined according to the fluorometric method of Hukkelhoven et al. (8; see also chapter 5). Dihydrodiol metabolites of BP, resulting from EH activity, were determined by the radiochemical method described (17; see also chapter 13). By this procedure both 7,8- and 9,10-dihydrodiol metabolites of BP can be quantitated. For both assays 20 hair follicles from each person were incubated in triplicate. DNA in the hair follicles was determined by the mithramycin technique (5; see also chapter 3). Activity was expressed as pmol phenolic metabolites/µg DNA/h or pmol dihydrodiol-BP/µg DNA/h. For each pair the ratio of phenolic as well as dihydrodiol metabolites of BP between the individuals was calculated.

RESULTS

In Table 1 the results are summarized for all 14 experiments. In this table the determinations of the two isomeric dihydrodiols of BP are also given separately. It should be noted that the formation of the 9,10-dihydrodiol metabolite exceeds the formation of the 7,8-dihydrodiol metabolite in all volunteers (data are not shown). However, as is shown in Fig. 1A, the intrapair ratios of

Table 1 Formation of BP-metabolites in hair follicles of pairs of individuals

pair of volunteers	A	B	C	D	E
I/II	1.51	1.38	1.34	1.36	1.36
III/IV	2.03	0.81	0.83	0.82	1.22
V/VI	1.52	1.24	0.91	1.08	1.08
V/VII	3.40	1.37	1.23	1.30	1.30
VIII/IX	2.75	1.79	1.79	1.79	1.79
IX/X	1.11	1.02	0.99	1.01	1.01
I/II	1.08	1.56	1.34	1.45	1.45
XI/XII	1.26	1.14	1.39	1.27	1.27
XIII/III	2.74	0.95	1.00	0.98	1.02
XIV/XV	1.01	1.34	1.95	1.65	1.65
XVI/XVII	2.27	0.51	0.75	0.62	1.61
XIV/XII	2.13	1.09	1.13	1.11	1.11
VII/XVIII	1.03	0.85	0.83	0.84	1.19
XIX/XX	2.21	1.20	1.48	1.34	1.34

(A) intrapair ratio of phenolic metabolites; (B) intrapair ratio of 7,8-dihydrodiol-BP; (C) intrapair ratio of 9,10-dihydrodiol-BP; (D) intrapair ratio of 7,8- + 9,10-dihydrodiol-BP; (E) intrapair ratio of 7,8- + 9,10-dihydrodiol-BP (highest activity in numerator, lowest activity in denominator).

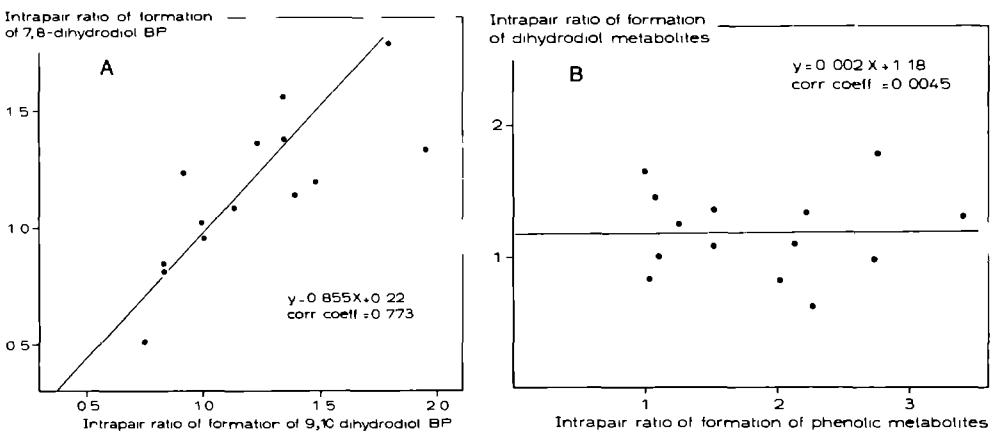

Fig. 1 (A) Relationship between intrapair ratio of 7,8-dihydrodiol-BP and 9,10-dihydrodiol-BP formation.
(B) Relationship between intrapair ratio of 7,8- + 9,10-dihydrodiol-BP formation and BP-phenol formation.

7,8-dihydrodiol-BP and 9,10-dihydrodiol-BP do correlate well within each pair. Furthermore, from Table 1 it may be concluded that the mean intrapair difference in formation of phenols exceeds the difference in formation of dihydrodiols (mean intrapair ratio of phenol formation is 1.86 ± 0.76, mean intrapair ratio of 7,8- + 9,10-dihydrodiol formation is 1.31 ± 0.24). This is also illustrated by the fact that for the dihydrodiol metabolites the intrapair ratio was never >2, while for the phenolic metabolites this occurred in 50% of the cases. Fig. 1B shows the correlation between phenol and dihydrodiol formation in each pair. As can be concluded from the regression analysis there exists virtually no correlation between formation of phenols and dihydrodiol metabolites of BP in human hair follicles.

DISCUSSION

Human hair follicles have been suggested as a biopsy tissue from which it is possible to predict an individual's overall ability to activate PAH (6-8,16). The question that remains to be answered is which enzymatic parameters are important and whether or not other biochemical end-points, such as DNA-binding, can serve as a standard for determination of individual susceptibility to carcinogens.

In this study we have found that the interindividual variation in phenol formation exceeds the interindividual variation in dihydrodiol formation, using human hair follicles as biopsy tissue and BP as model compound for PAH. Low interindividual variation for EH has been reported earlier for human lung microsomes (12), in human colonic biopsies (4) and in cultured human fibroblasts (11), while for AHH greater interindividual variation has been found, especially in microsomal preparations from human lung, liver and placenta (13-15). An explanation for the smaller interindividual variation in dihydrodiol formation compared to phenol formation could be that induction of EH *in vivo* or *in vitro* has never convincingly been reported for human tissues, while the inducibility of AHH in various human tissues is well documented (1,2). Moreover, it has been shown that in human bronchial epithelial cells (9; see also

chapter 14) and in hair follicle and epidermal keratinocytes (10; see also chapter 13) pre-incubation with benz(a)anthracene leads to increased BP-metabolism towards phenols, but does not affect BP-metabolism towards dihydrodiols.

In the present study it is further demonstrated that formation of phenolic metabolites of BP does not correlate with formation of dihydrodiol metabolites. This is not surprising in view of the complex metabolism of BP, which is converted to at least 30 metabolites including phenols, epoxides, quinones, dihydrodiols, diol epoxides and a range of water-soluble conjugates (3). Thus, if the concentration of phenolic or dihydrodiol metabolites plays an important role in the biological effects of PAH in the cell, measurement of these two types of metabolites can not be interchanged in assessing an individual's susceptibility to the carcinogenic action of PAH.

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PART 4

CORRELATION OF HUMAN AND MURINE BENZO(a)PYRENE METABOLISM

13

Formation of Metabolites of Benzo(a)pyrene in Cultured Human and Murine Skin Cells

ABSTRACT

The formation of the 7,8- and 9,10-dihydrodiol metabolites of benzo(a)pyrene (BP), which are believed to play a role in the chemical induction of tumors, was investigated in cultures of human and murine origin. It was found that cultures of mouse (strain C3Hz) epidermal and skin fibroblastic cells showed inducible BP metabolism towards dihydrodiol metabolites, after pre-incubation with benz(a)anthracene (BA). This was consistent with the increased *in vivo* formation of dihydrodiol metabolites of BA after injection with 3-methylcholanthrene. In contrast, in human cell cultures the metabolism of BP to the dihydrodiol metabolites was not enhanced after pre-exposure to BP. This was the case in low-passage skin fibroblasts, primary epidermal skin cells, and primary keratinocytes from hair follicles. Moreover, other inducers of microsomal oxygenases, such as phenobarbital and 3-methylcholanthrene, were also unable to increase BP metabolism towards the dihydrodiol compounds. However all cell cultures formed larger amounts of phenolic BP-metabolites after pre-exposure to BA. In view of these results, obtained using *in vitro* human and murine model systems, we may conclude that human and murine skin cell culture systems respond differently to pre-treatment with inducers of microsomal monooxygenase with respect to the metabolism of BP to reactive dihydrodiol metabolites. The possible implications for the human *in vivo* situation are discussed.

Polycyclic aromatic hydrocarbons (PAH) are widespread environmental pollutants and potential carcinogens that are metabolised to a large spectrum of organic solvent-soluble metabolites as well as to water-soluble conjugates. Some of the metabolites of PAH produced in the body are more potent carcinogens *in vivo* than the original hydrocarbon. Others show little or no carcinogenicity and can be considered as detoxification products. Benzo(a)pyrene (BP) is the most widely studied model compound of PAH. Although several different metabolic intermediates have been suggested as the ultimate carcinogenic form of BP, several recent reports point to the trans-7,8-dihydrodiol-9,10-epoxides of BP (2,13,16, 22,26,29). These metabolites are formed from the trans-7,8-dihydrodiol of BP. The amount of this crucial diol-compound present in the cell is the result of the balance between activating and detoxifying enzyme reactions. Therefore, it is hard to assess the significance of individual enzyme activities involved in PAH-metabolism.

Aryl hydrocarbon hydroxylase (AHH) is the first enzyme involved in the metabolic transformation of PAH (5). Because this enzyme is responsible for the production of reactive epoxides from PAH, AHH has been considered as a key enzyme in the chemical induction of tumors (19,28). Moreover, attempts have been made to correlate differences in inducibility of AHH with susceptibility both in mice (15) and in humans (1,14,18,23). Because of the importance of the dihydrodiol metabolites of BP as precursors of the carcinogenic dihydrodiol-epoxides, we have investigated the formation of the 7,8- and 9,10-dihydrodiol metabolites of BP in cultures of human and murine origin. Attention was focused on the effect of inducers of AHH on the formation of these crucial intermediates of BP metabolism. Because epithelial cells are the target cells for many PAH *in vivo* we used short-term cultivated epithelial cells of both species. Human and murine low-passage skin fibroblasts, essentially free of epidermal cells, served as normal mesenchymal cells.

MATERIALS AND METHODS

Chemicals

[G-^{3}H] BP (specific activity 19 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, UK, and was purified chromatographically before each experiment (solvent system: toluene : ethanol (9 : 1)). The radioactive BP was eluted from the silica gel plate with ethylacetate. Reference polynuclear aromatic hydrocarbon metabolites were donated by the National Cancer Institute Chemical Repository at the IIT Research Institute, Chicago, IL. NADPH was obtained from Sigma (Saint Louis, Mo., USA). Fetal calf serum, Minimal Essential Medium (with Earle's salts), penicillin/streptomycin and glutamine were purchased from Gibco (Glasgow, UK). For thin layer chromatography, $F60_{254}$ silica gel plates (Merck, Darmstadt, FRG) were employed. Kodak X-ray XR-2 films were used for autoradiography. EN^{3}HANCE (New England Nuclear, Boston, Mass., USA) was used as an autoradiography enhancer. A mixture of 3g 2,5-diphenyloxazol and 0.2 g 2,2-phenylenebis (4-methyl-5-phenyloxazole), both purchased from Merck,and dissolved in 1 l technical toluene was used as scintillation fluid.

Cell cultures

Skin from newborn C3Hz mice, from which subcutaneous fat was removed, was cut into approximately 1 mm^2 pieces and placed epidermal-side up in plastic Falcon flasks. They were incubated in Minimal Essential Medium supplemented with 15% fetal calf serum, 2 mM glutamine, penicillin (50 I.U. per ml), and streptomycin (50 µg per ml) at 37°C in a 95% air, 5% CO_2 atmosphere. After clear outgrowth of epithelial and fibroblastic cells could be observed, fibroblasts were selectively removed by trypsin treatment (0.35% trypsin, 0.002% EDTA in Tyrode (137 mM NaCl, 2.7 mM KCl, 0.4 mM $NaH_2PO_4.H_2O$, 11,9 mM $NaHCO_3$, 11.1 mM glucose, pH 7.2)). These fibroblasts were selectively cultured till confluency. No attempt was made to passage the epithelial cells to other flasks. Growth of almost confluent epithelial cell sheets required about one month. Human epidermal and fibroblastic cells were obtained in the same manner. Skin biopsies were obtained from the shoulder-blade of healthy adult volunteers. Human keratinocytes were obtained by culturing human hair follicles on extracellular matrix (bovine eye lens capsules; see Hukkelhoven et al. (9; see also chapter 2)) using an especially constructed culture dish (Epicult, made available by Sanbio B.V., Nistelrode, The Netherlands) as described by Weterings et al. (25).

Induction of AHH-activity in cultures

Aryl hydrocarbon hydroxylase was induced in primary epithelial cells, fibroblastic cells of the first subculture, and in human hair follicle keratinocytes by exposure to culture medium containing 10 µM benz(a)anthracene in dimethylsulfoxide (DMSO). Control cultures were treated with DMSO alone. The final DMSO concentration used in induced and control cultures was 0.1%. The hydrocarbon exposure was carried out for 16 h. The culture medium was identical to that previously described in the 'cell cultures' section, except that 15% heat inactivated fetal calf serum (30 min, 56°C) was used. After exposure the cultures were rinsed four times with Tyrode solution. Human skin and hair follicle keratinocytes grown *in vitro* were also incubated with other inducers of aryl hydrocarbon oxygenases, such as 3-methylcholanthrene and phenobarbital (10 µM).

Measurement of BP-metabolites

Cultures were analyzed for AHH-activity by adding ^{3}H-labeled BP (3 µCi) and 2 mmol NADPH in 1 ml culture medium (containing 15% heat inactivated fetal calf serum). After incubation for four h at 37°C the cells were scraped from the

surface. In the cultures of hair follicle keratinocytes whole lens capsules together with the hair follicles and the outgrowth of the hair follicles were removed from the surface. Lens capsules without cells were incubated as blanks. Cells and medium were extracted three times with two volumes of ethylacetate. The combined organic phases were evaporated to dryness under a nitrogen stream. The residue was redissolved in 10 µl etnanol, applied to thin layer plates and chromatographed in a toluene-ethanol mixture (9 : 1) together with reference metabolites. The reference compounds were visualized with UV-light. After spraying the plates with autoradiography enhancer, the radiometabolites were examined autoradiograpically by exposure of the plates to X-ray film. For quantification of the results, the areas of radioactivity were scraped off the plates and transferred to counting vials which contained 1 ml of methanol. For liquid scintillation analysis, 10 ml of scintillation fluid was added. Phenolic BP-metabolites were measured as described earlier (11; see also chapter 5).

In vivo studies

C3Hz Mice were pretreated by a single intraperitoneal injection of arachis oil (0.5 ml), or 3-methylcholanthrene in arachis oil (100 mg/kg). After sacrifice, skin samples were finely minced and incubated for 1 h in 1 ml of a mixture containing 50 mM Tris-HCl buffer, pH 7.5, 0.3 M sucrose, 100 units penicillin/ml, 2 mM NADPH, 3 mM $MgCl_2$ and 3 µCi[^{3}H]-BP. Further procedures were identical to the methods described for the cultures.

Determination of DNA

DNA was determined by the mithramycin technique (7), with modifications according to Hukkelhoven et al. (10; see also chapter 3). Calf thymus DNA was used as a reference standard in the calculation of the DNA content.

RESULTS

Incubation of epithelial and fibroblastic cultures of human and murine origin results in the formation of two major radiolabeled compounds. After separation on thin layer plates and co-chromatography with several known BP-metabolites, they can be identified as trans-9,10-dihydrodiol- and trans-7,8-dihydrodiol benzo(a)pyrene.

Fig. 1 shows an example of an autoradiograph obtained after incubation of human keratinocytes with [^{3}H]BP. Pre-incubation with BA does not result in an increased metabolite formation. In fact, the amount of dihydrodiols formed is slightly reduced. Human skin epithelial cells and fibroblasts were also not able to metabolize BP to a greater extent after pre-exposure to BA. In contrast, mouse epithelial and fibroblastic cells were inducible with respect to the formation of the 7,8- and 9,10-dihydrodiols of BP after pretreatment with BA.

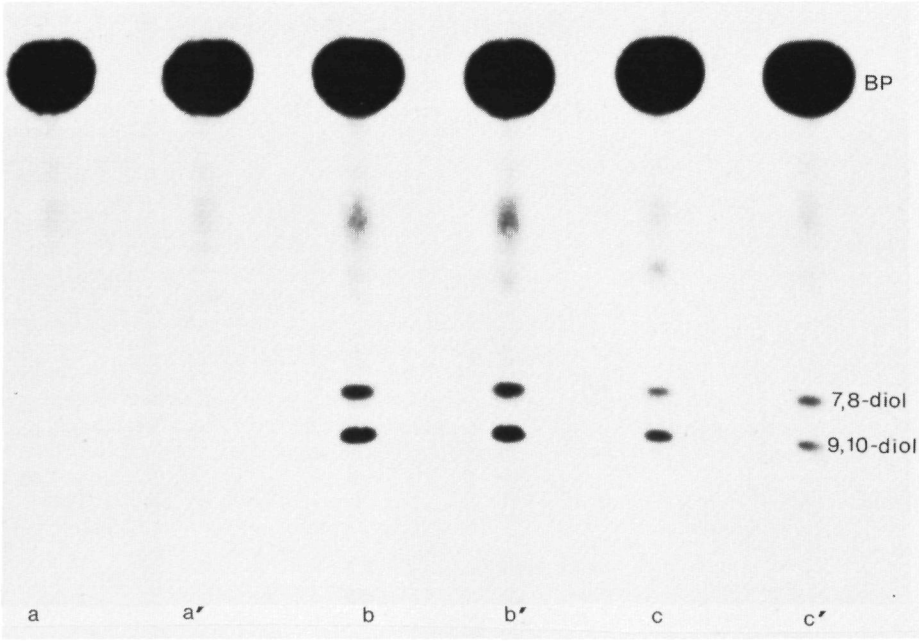

Fig. 1 Autoradiograph of a chromatogram of benzo(a)pyrene metabolites extracted after incubation of [^{3}H]BP with human keratinocytes from hair follicles cultured on bovine eye lens capsules. a, á: bovine eye lens capsules without cells; b, b́: human keratinocytes; c, ć: human keratinocytes after pre-incubation with benz(a)anthracene (10 µM).
Abbreviations: 7,8-diol = 7,8-dihydro-7,8-dihydroxyBP
9,10-diol = 9,10-dihydro-9,10-dihydroxyBP

The induction ratio of each metabolite was calculated as the amount of metabolite per µg DNA formed in the BA-treated culture, divided by that in the control culture. The results for the various cultures and the *in vivo* experiments are summarized in Table 1. It can be concluded that all murine systems show inducible BP-metabolism towards the dihydrodiol-metabolites of BP. Alternatively, none of the tested cultures of human cells are able to metabolize BP to these intermediates to a greater extent after pre-exposure to BA. The same results were obtained when human keratinocytes were incubated with phenobarbital and 3-methylcholanthrene (results not shown).

Table 1 Induction of formation of metabolites of BP *in vitro* and *in vivo* by pre-treatment with BA (10 µM).

system	7,8-dihydro-diol BP	9,10-dihydro-diol BP	phenolic BP-metabolites
mouse skin *in vivo*	2.6 ± 0.4	2.8 ± 0.6	8.2 ± 1.9
mouse skin epithelial cells	3.2 ± 0.6	3.3 ± 0.8	4.7 ± 1.2
mouse skin fibroblasts	4.2 ± 0.8	4.3 ± 1.0	5.1 ± 1.5
human skin epithelial cells	0.9 ± 0.2	1.0 ± 0.3	3.8 ± 0.9
human skin fibroblasts	1.1 ± 0.2	0.9 ± 0.2	4.1 ± 1.0
human keratinocytes (from hair follicles)	0.6 ± 0.1	0.7 ± 0.2	4.3 ± 1.4

Figures represent ratios between induced and control amounts of metabolites formed and are the mean of 5 determinations.

As analyzed by the fluorometric AHH-assay all human and murine cell-types metabolized BP to phenolic metabolites to a greater extent after pre-exposure to BA.

DISCUSSION

For practical purposes, it is obviously of great importance to establish whether chronic exposure of human tissues to low levels of PAH will result in metabolic shifts which favour the formation of carcinogens. Unfortunately, two problems arise in providing a definitive answer to this question: Firstly it is difficult to define the precise metabolite of importance, and to develop specific assays for these substances; Secondly, ethical prohibitions preclude *in vivo* experiments of this type on human subjects.

In most animal studies AHH-inducibility is measured after administration of the inducing agent in living animals (3,24). Kouri et al. concluded that AHH-inducible inbred strains of mice were more susceptible to 3-methylcholanthrene-induced subcutaneous tumors than were their non-inducible counterparts (15). These results were obtained by measurement of the fluorescent phenol

metabolites of BP (21). These metabolites have only limited carcinogenic potency (6,12,27), with the possible exception of the 11-OH and 2-OH BP derivatives. However, these have never been isolated as metabolites of BP, and whether they are formed *in vivo* remains to be determined. In mutagenesis test systems these compounds also show low activity both in microbial systems (20) as well as in mammalian cells (8). On the other hand phenols are rearrangement products of metabolically formed benzo(a)pyrene epoxides which are believed to be highly mutagenic. Moreover, it has been established that the level of AHH generally parallels total BP-metabolism (30). Of all the dihydrodiol, phenol, and quinone metabolites of BP the trans-7,8-dihydrodiol has been shown to be the most active precursor of mutagenically-active compounds in V79 hamster cells (8) as well as in human cells (17).

We used C3Hz mice, a strain that has been classified as aromatic hydrocarbon responsive or AHH-inducible (8), to measure the formation of two important PAH dihydrodiol metabolites (7,8- and 9,10-dihydrodiolbenzo(a)pyrene) after *in vivo* induction with 3-methylcholanthrene. From Table 1, it can be concluded that pre-treatment of C3Hz mice with 3-methylcholanthrene does indeed result in increased metabolism of BP to the 7,8- and 9,10-dihydrodiol metabolites in skin.

To evaluate the validity of extrapolation from *in vitro* data to *in vivo* conclusions, especially necessary for the human situation, experiments were set up to investigate whether this ability to metabolize BP to a greater extent to diol-metabolites after induction of AHH, was maintained in cultured skin cells of C3Hz mice. We found that this was indeed the case, both in primary epidermal cells and in low-passage skin fibroblasts. Therefore, these experiments suggest that the factors controlling the formation of the dihydrodiol compounds of BP in skin from adult mice that received 3-methylcholanthrene, also control the formation of these diols in epidermal and fibroblastic mouse cells in culture after pre-treatment with AHH-inducers.

In view of the high biological activity of the dihydrodiol-derivatives of BP, and the widespread occurrence of BP as a chemical pollutant in the environment and in certain foods, it is of great importance to know the effects of chemically defined AHH-

inducers on the formation of dihydrodiols of BP in human tissues. Unfortunately, for the human situation *in vivo* these data do not exist. Therefore, we established cultures of both primary epidermal cells and low-passage fibroblasts as well as of primary keratinocytes cultured from human hair follicles to investigate these effects *in vitro*. Pre-treatment with AHH-inducers in all three human culture systems did not result in increased BP-metabolism towards dihydrodiols. In fact, in human hair follicle keratinocytes the amounts of diol-metabolites were decreased to 60-70% of those of the control cultures. This is in accordance with the results of Fox et al. (4), who noted that pre-exposure of human epithelial cell cultures to mixtures of PAH resulted in a decrease in the amounts of carcinogen metabolized to dihydrodiols.

Phenolic BP-metabolites (which result from the action of AHH) can not be adequately quantitated from t.l.c. plates due to their relatively large unstability. However, using a fluorometric AHH-assay (11), we have found that all three human culture systems employed in this report - as well as murine skin epidermal and fibroblastic cells - are inducible with respect to the formation of phenolic BP-metabolites upon pre-exposure to BA.

The C3Hz mouse model, as described in this chapter, has shown that the effects of inducers of AHH with respect to the formation of the crucial diol-metabolites of BP, are comparable *in vivo* and *in vitro*. When the results for the human culture models presented in this study are extrapolated to the *in vivo* situation, one has to conclude that exposure of man to AHH-inducers does not result in increased dihydrodiol formation of PAH. Therefore, in humans the risk to obtain cancer upon exposure to PAH may not necessarily depend on AHH-inducibility, as has been suggested for certain strains of mice.

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14

Differences in Benzo(a)pyrene Metabolism between Cultured Human and Murine Bronchial Cells after Pre-Treatment with Benz(a)anthracene

ABSTRACT

Primary cultures of human and murine (strain C3Hz) bronchial epithelial cells were pretreated with benz(a)anthracene (BA) (10 μM). 16 h later the formation of phenolic as well as dihydrodiol metabolites of benzo(a)pyrene (BP) was measured. Whereas murine cultures showed enhanced metabolism towards both phenolic and dihydrodiol compounds, in the human cultures only phenolic BP-metabolites were increased. In view of their precursor role in the formation of biologically active diol-epoxides, formation of dihydrodiol-derivatives can be considered as a key factor in determining susceptibility to polycyclic aromatic hydrocarbons (PAH)-induced neoplasia. Therefore the observations of this study indicate that animal model systems for PAH carcinogenesis in man have to be selected on the basis of comparable metabolite patterns.

INTRODUCTION

It is generally accepted that mutagenic and carcinogenic effects of polycyclic aromatic hydrocarbons (PAH), of which benzo(a)pyrene (BP) is the most widely studied prototype compound, require metabolic activation of the inactive procarcinogens (5,13). Metabolism of PAH towards biologically active intermediates is mediated by microsomal enzymes, especially aryl hydrocarbon hydroxylase (AHH) and epoxide hydrolase (EH). AHH has been shown to be inducible in a variety of cell-types after exposure to PAH (14). A lot of research has implicated diol-epoxides as the major reactive intermediates in BP-metabolism (2,3,18). For this reason measurement of

formation of dihydrodiol-metabolites of BP, the direct precursors of the diol-epoxides, is of great importance in analyzing biological effects of PAH. In this study it is reported that cultured mouse (strain C3Hz) bronchial cells and the corresponding human *in vitro* model, show different BP metabolism towards dihydrodiol derivatives after pretreatment with benz(a)anthracene (BA).

MATERIALS AND METHODS

Chemicals

[G-^{3}H]BP (specific activity 19 Ci/mmol) was obtained from the Radiochemical Centre, Amersham (UK), and purified chromatographically before each experiment. BP and BA were purchased from Aldrich, Beerse (Belgium) and reference PAH metabolites were a gift from the IIT Research Institute, Chicago. NADPH was obtained from Sigma (St. Louis, MO). Fetal calf serum, Minimal Essential Medium (with Earle's salts, MEM) and glutamine were purchased from Gibco, Glasgow (UK), gentamycin sulfate was from Schering, Kenilworth (UK). For thin-layer chromatography, $F60_{254}$ silica gel plates (Merck, Darmstadt, FRG) were employed. Kodak X-ray XR-2 films were used for autoradiography. EN^{3}HANCE (New England Nuclear, Boston) was used as an autoradiography enhancer. A mixture of 3 g 2,5-diphenyloxazole and 0.2 g 2,-2´-p-phenylenebis(5-phenyloxazole) both purchased from Merck, Darmstadt was used as scintillation fluid (when dissolved in 1 l toluene).

Cell cultures

Human bronchial tissue was obtained at bronchoscopy (for diagnostic purposes) or immediate autopsy. Only macroscopically unaffected tissue was used. The tissue was transported to the laboratory in ice-cold MEM supplemented with 10% fetal calf serum and 10 µg/ml gentamycin sulfate. Explants of 1-2 mm^2 were put in prewetted plastic Falcon flasks (25 cm^2) and allowed to stick to the bottom for 3 h at 37°C. Then 3 ml culture medium (MEM supplemented with 15% fetal calf serum, 2 mM glutamine and 10 µg/ml gentamycin sulfate) was added. Incubation was performed in a humidified atmosphere of 5% CO_2 in air at 37°C. The medium was replaced every 3 days. Cellular outgrowths consisted almost entirely of polygonal-shaped epithelial cells. Some of them showed ciliary activity. In some cultures spindle-shaped fibroblasts could be observed. These cultures were not used. Bronchial cells established from adult male C3Hz mice were cultured in the same way. After 3 weeks in culture, when epithelial cell sheaths had grown to a distance of 2 cm from the explants, BP-metabolism studies were started. Fig. 1 shows a phase contrast microscopical picture of human bronchial epithelial cells in culture.

Induction of enzyme activity

Cultures were pretreated by exposure for 16 h to culture medium containing 10µM BA in dimethylsulfoxide (DMSO) (final DMSO concentration 0.1%). The culture medium contained 15% heat-inactivated (30 min, 56°C) fetal calf serum. After exposure the cultures were rinsed 4 times with 0.9% NaCl solution.

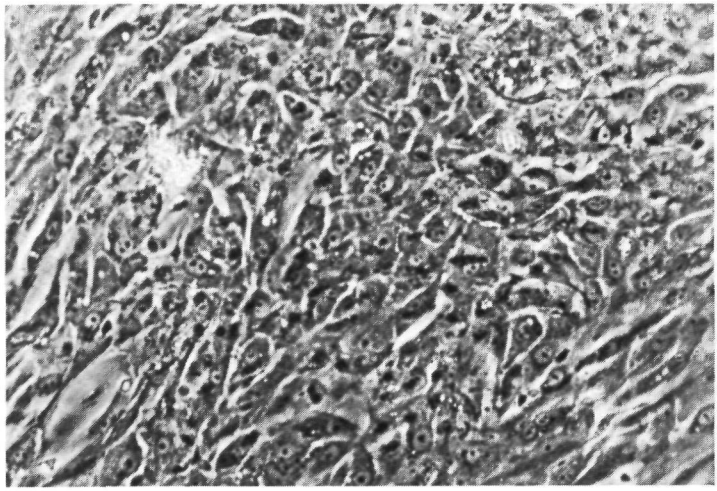

Fig. 1 Phase contrast microscopical picture of human bronchial epithelial cells in culture. The black spots are bundles of cilia.

Measurement of BP-metabolism towards dihydrodiols

BP-metabolism in the cultures was measured by adding [^{3}H]BP (30 µCi, mixed with unlabeled BP to a final concentration of 25 µM) and 2 mmol NADPH to 1.5 ml 50 mM Tris-HCl, pH 8.5 (containing 0.1 M sucrose and 3 mM $MgCl_2$). After incubation for 2 h at 37°C the cells were scraped from the surface. Cells and medium were extracted 3 times with 2 vols. of ethylacetate. The combined organic phases were evaporated to dryness under a nitrogen stream. The residue was redissolved in ethanol, applied to thin-layer plates and chromatographed in a toluene-ethanol mixture (9 : 1) together with reference metabolites. The reference compounds were visualized with UV-light. After spraying the plates with autoradiography enhancer, the radiometabolites were examined autoradiographically by exposure of the plates to X-ray film. For quantification of the results, the areas of radioactivity were scraped off the plates and transferred to counting vials, which contained 1 ml of methanol and 10 ml of scintillation fluid.

Measurement of aryl hydrocarbon hydroxylase

AHH in the cultures was measured according to the fluorometric method described earlier (7). Incubation was performed for 2 h. See also chapter 5.

Determination of DNA

DNA was determined by the mithramycin technique (6) with modifications according to Hukkelhoven et al. (8). See also chapter 3.

RESULTS

Incubation of human and murine bronchial cultures with [^{3}H]BP results in the formation of three major radiolabeled compounds which, after cochromatography with several known BP metabolites, can be identified as trans-4,5-dihydrodiol-BP, trans-7,8-dihydrodiol-BP and trans-9,10-dihydrodiol-BP. In whole cells the amount of phenolic BP metabolites is relatively low (see e.g. 12). Therefore, and because of the relative instability of BP-phenols on t.l.c., the formation of these metabolites was not calculated from the thin-layer plates but from the more sensitive fluorometric AHH-assay. The induction ratio of each metabolite was calculated as the amount of metabolite/µg DNA/h formed in the BA-treated cultures divided by that in the control cultures. The results are summarized in Table 1. It is obvious that in the mouse cells pretreatment with BA results in increased BP metabolism towards phenolic products and dihydrodiols, while human bronchial cells are only inducible with respect to the phenolic metabolites.

Table 1 Induction ratios of different BP metabolites after pretreatment with BA (10 µM).

culture system	4,5-diol*	7,8-diol	9,10-diol	phenols
human bronchial cells	0.9 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	3.8 ± 0.4
C3Hz mouse bronchial cells	3.6 ± 0.3	3.9 ± 0.4	3.8 ± 0.4	4.3 ± 0.6

Each value represents the mean of four determinations.
**The formation of 4,5-diolBP is very low compared to 7,8- and 9,10-diolBP.*

DISCUSSION

Biological effects of PAH are closely related to the steady-state level of mutagenic and carcinogenic metabolites. Most studies of activation and detoxification of PAH have focused on the liver microsomal monooxygenase system. However, arene oxides formed in the liver can probably not efficiently escape the organ to affect extrahepatic organs. Therefore, specific organ mutagenicity and

carcinogenicity might be directly related to PAH-metabolizing enzymes in the organs themselves. Several reports have indicated that broken-cell preparations have different metabolic routes than intact cells, with intact cells better simulating the *in vivo* situation (1,4). Moreover, species and even strain specific differences in carcinogen metabolism do exist (16,17).

To study metabolic responses after pre-treatment with BA in intact cells, we have used human and murine cell cultures of the bronchus, a target organ for PAH carcinogenesis. It was found that the crucial dihydrodiol metabolites of BP were not induced in human cultures, in contrast to the situation in murine cells, thus reflecting a basic difference in carcinogen metabolism between both species. The same differences between both species have been obtained with cultured epidermal cells (including human hair follicle keratinocytes) and skin fibroblasts (chapter 13).

Kouri et al. (11) showed that susceptibility to subcutaneous tumors induced by 3-methylcholanthrene was correlated to AHH inducibility in various strains of mice. Attemps to establish such a relationship in humans resulted in conflicting reports (10,15). All AHH-assays in these studies are based on the measurement of only one type of PAH metabolite, the phenols, which have only limited carcinogenic potency (9). The finding that dihydrodiol metabolites of BP are not induced in human bronchial cell cultures after pre-exposure to BA, in contrast to the murine cells, can form a possible explanation for the absence of a clear correlation between AHH-inducibility and risk for chemical-induced cancer in humans. In establishing *in vitro* animal systems to study the various processes involved in human carcinogenesis it is therefore important to select them on the basis of comparable metabolite patterns.

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PART 5

INDUCTION OF ARYL HYDROCARBON HYDROXYLASE IN HUMAN TISSUES IN VIVO

15

The Use of Liposomes in the Topical Application of Potentially Aryl Hydrocarbon Hydroxylase Inducing Substances

ABSTRACT

The applicability of liposomes as a drug carrier system for potential use in local delivery of aryl hydrocarbon hydroxylase-inducing substances in man, was investigated. Increased local concentrations after application of corticosteroids in liposomes have been described (Mezei and Gulasekharam, Life Sci. 26: 1473, 1980). However, an increased biological effect has never been demonstrated. In order to verify this biological effect we used the hamster flank organ model, which represents a test model of local action of androgens. Therefore the effect of topical application of the androgen 5α-dihydrotestosterone, both encapsulated in liposomes and solved in acetone, was evaluated in the hamster flank organ. Under the experimental conditions used, unequivocal advantages of the liposome system over application in acetone could not be demonstrated. Hence, the liposome system with encapsulated aryl hydrocarbon hydroxylase-inducing substances is not markedly more efficient for determining interindividual differences in aryl hydrocarbon hydroxylase-inducibility.

INTRODUCTION

To achieve detection of interindividual differences in aryl hydrocarbon hydroxylase (AHH)-inducibility *in vivo* in hair follicles it was considered that liposomes (phospholipid bilayer vesicles) might be an effective application form of AHH-inducing compounds. As a model for studying the potential of this delivery system we applied liposome-encapsulated steroids to sebaceous

structures in hamsters and evaluated the local and systemic effects. This system has the advantage that conclusions can be extended to aspects of general drug delivery and resulting biological effects.

The therapeutic value of topically applied drugs depends on their optimal concentration at the target organ. Liposomes were used as carriers for the topical application of triamcinolone acetonide (3,4). After five days of application these authors measured a drug concentration in the epidermis and dermis that was 4 times higher than that obtained using a control ointment, whereas urinary excretion was diminished. Therefore their results indicate that the use of liposomes decreased the percutaneous absorption of the drug. They claimed a sustained (gradual) release of the drug as a consequence of the direct interaction of the drug releasing vesicles with cells at the target site. This effect may be similar to the one that has been well documented for systemic applied liposomes (5). In the case of intact skin the above claim implies the passage of 0.2 - 1.0 µm particles through the densily packed, fully keratinized, horny layer, with barrier functions responsible for preventing the diffusion of many locally applied drugs into the depth of the skin.

To find further evidence that liposomes have a selective drug delivery potential for cutaneous application, we applied 5α-dihydrotestosterone (DHT) to hamster flank organs. (Note: DHT is not a AHH-inducing steroid but is merely used as a model compound to test the potential of the liposome delivery system.) The flank organs are sebaceous structures located one on each flank and, like the sebaceous gland in man and other species, they are androgen dependent. In the mature male hamster the organ measures approximately 6 mm, is heavily pigmented and is covered with coarse dark hairs, in the female it is about 2 mm in diameter, lightly pigmented, with few dark hairs. The active androgen is DHT and its application to the flank organ of the female hamsters induces an increase in its diameter and size (6,7). The system allows verification of the biological effect of androgens and can be used to assess the merits of drugs and AHH-inducing substances encapsulated in liposomes. In comparison with application in traditional formulations, assessment of the biological effects might be expected to be a more accurate means for the evaluation of the efficiency of

the dosage forms than dermal concentration measurements.

MATERIALS AND METHODS

Preparation of liposomes

Liposomes were prepared from DL-α-palmatoyl-phosphatidylcholine and cholesterol (Sigma Chem. Co., St. Louis, Mo., USA) with a molar ratio of 1.1 : 0.5, as described by Mezei and Gusalekharam (3). A total of 30 mg lipids together with 5 mg 5α-dihydrotestosterone (DHT) was dissolved in 20 ml chloroform in a round bottom flask. The solvent was evaporated at 37°C and the liposomes were produced by dispersion of the lipid film with 2 ml 8 mM $CaCl_2$-solution. The liposomes were centrifuged (8,000 g, 10 min) and washed with 5 ml 8 mM $CaCl_2$-solution. This washing procedure was repeated three times. After the last centrifugation step the liposomal pellet was redissolved in 2 ml 8 mM $CaCl_2$-solution.

In a separate experiment, using ^{3}H-DHT (Amersham, UK) this washing procedure was proven to be sufficient to remove all of the unincorporated DHT. The diameter of the liposomes was about 1 µm. 20 µl of the liposomal suspension, prepared as described above, contained 40 µg DHT. A liposomal suspension containing 20 µg DHT was prepared by diluting the original suspension. To achieve a DHT-concentration of 4 µg DHT in 20 µl liposomal suspension, new liposomes were prepared using 0.5 mg DHT. For control experiments liposomes without DHT were also prepared. Each tenth day the liposome suspensions were freshly prepared.

Flank organ test

The hamster flank organ test was carried out as described earlier (7,8). The female hamsters were separated into six groups of five animals and treated according to the following scheme:

group I : 4 µg DHT (dissolved in acetone)
group II : 20 µg DHT (dissolved in acetone)
group III: 40 µg DHT (dissolved in acetone)
group IV : 4 µg DHT (encapsulated in liposomes)
group V : 20 µg DHT (encapsulated in liposomes)
group VI : 40 µg DHT (encapsulated in liposomes)

The animals were treated once daily (five days a week). After 28 days of treatment the flank organs were excised for morphometrical and histochemical examination.

Histology

For histological and morphometrical evaluations squares of shaven skin containing the flank organ were pinned in a flat position on cork plates, quickly frozen in liquid nitrogen (-190°C) and stored in a freezer at -90°C until further use. Cranio-caudal cross-sections (8 slides with 3 sections each of approx. 10 µm thickness per organ) were cut through the center of the organ with a Walter-Dittes Cryostat at -25°C. The sections were stained routinely with Paragon® (multiple stain for frozen sections) and with haematoxylin-eosin.

For morphometrical analysis a microscopical image of the organ was projected on a sheet of paper, at a fixed magnification, and drawn. The length of the organ was measured: the borderlines of the organ were drawn just outside the outer, larger sebaceous gland (compared with the small sebaceous glands of the

normal skin). The drawings of the sebaceous glands were cut out with a swivel knife (Ulano®, Switzerland) and the paper was weighed. This method has been published previously (2). Results are reported as mean ± S.E. Data were analyzed by using Student's t-test to determine the difference between two means for paired observations on the basis of a P-value < 0.05 ($\alpha = 0.05$).

RESULTS AND DISCUSSION

After topical application of 4 µg 5α-DHT to the left flank organ of female hamsters the diameter of the pigmented spot is doubled to a value of 6 mm (Fig. 1). The diameter of the pigmented spot did not increase with higher doses. The form of application (a solution in acetone or a suspension in the form of liposomes) did not influence this phenomenon. The application of 20 µg DHT and

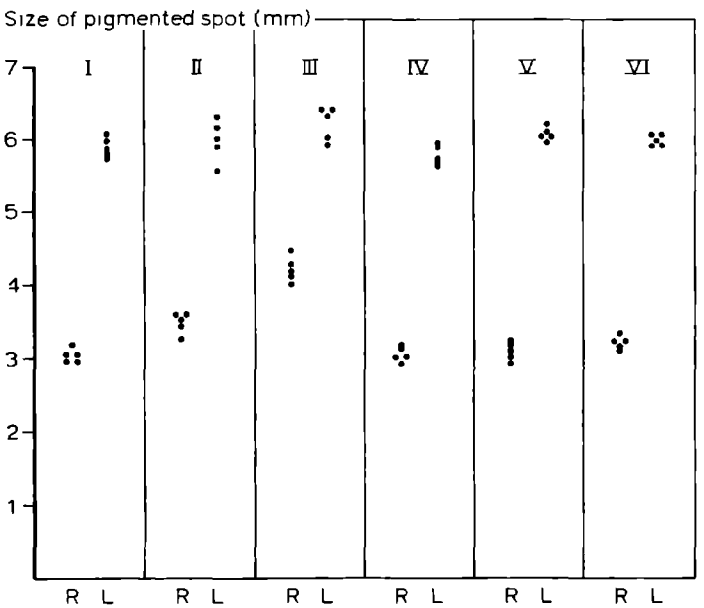

Fig. 1 Diameter of the left (L, treated) and right (R, control) pigmented spot of groups of 5 female hamsters in relation to the concentration of 5α-dihydrotestosterone (DHT) and the vehicle used.

I : 4 µg DHT (in acetone)
II : 20 µg DHT (in acetone)
III: 40 µg DHT (in acetone)
IV: 4 µg DHT (in liposomes)
V : 20 µg DHT (in liposomes)
VI: 40 µg DHT (in liposomes)

especially 40 µg DHT resulted in an increase in diameter of the pigmented spot of the contralateral organ (Fig. 1). This effect did not occur when DHT was encapsulated in liposomes.

The increase in size of the total sebaceous gland structure is a more specific indication of the stimulatory properties of androgens than the increase of the pigmented spot alone (2).

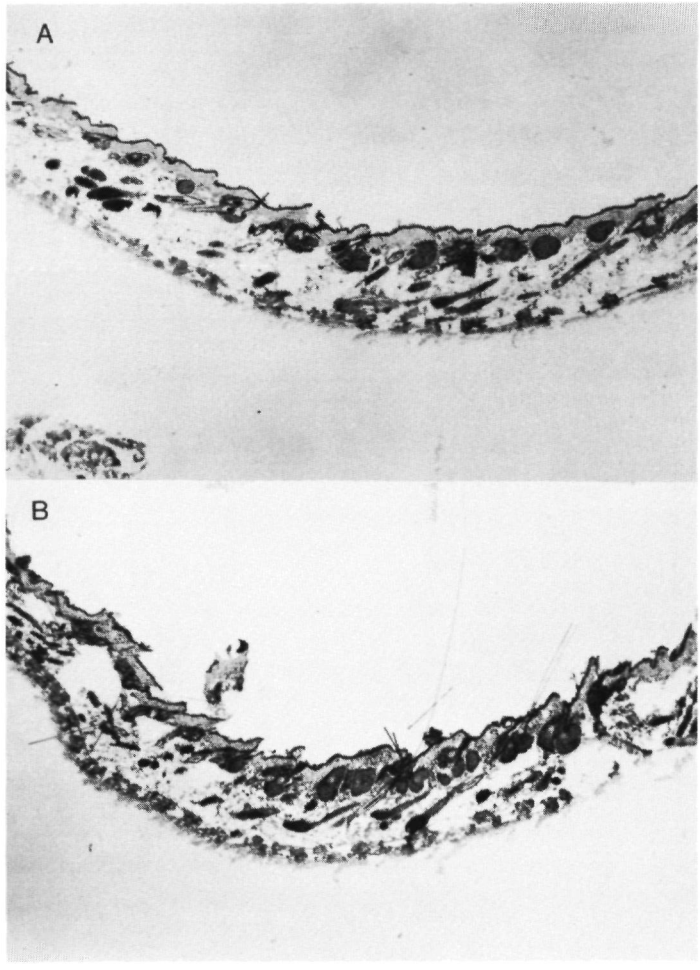

Fig. 2 Effect of the application of 5α-dihydrotestosterone (DHT) to the contralateral flank organ: (A) application of 40 µg DHT in liposomes. (B) application of 40 µg DHT in acetone.

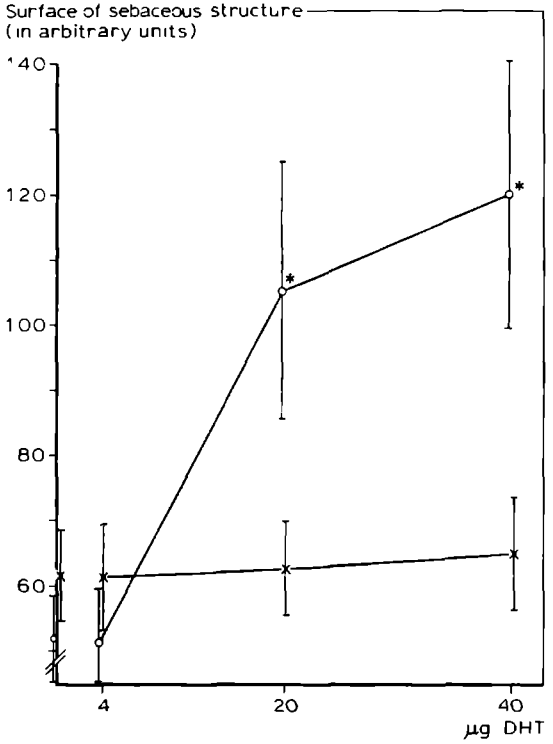

Fig. 3 Effect of increasing concentrations of 5α-dihydrotestosterone (DHT) on the size of the untreated control flank organ in relation to the various vehicles used: o - o: *acetone (results are expressed as mean ± S.D.)*
x - x: *liposomes*

* = *significant difference on the basis of* $\alpha = 0.05$.

Under the influence of DHT the female flank organ increases in size to resemble the male flank organ. The effect can be quantified by measuring the area of the sebaceous structure, and the extent of the increase will depend on the amount of the active agent reaching the target site.

The application of 20 and 40 µg DHT in acetone results in an increase in size of the sebaceous glands of the contralateral flank (Fig. 2 and 3). This increase is significant ($\alpha = 0.05$), both in comparison with the totally untreated flank organ (DHT-concentration = 0) and those of animals treated with 4 µg DHT in acetone or 20 or 40 µg DHT in liposomes. Since such an increase

can not be the result of a direct diffusion of the androgen from the skin surface to the sebaceous gland it must be indirect. It is highly likely that acetone allows DHT to reach the lower layers of the skin where it is absorbed in sufficient amount to affect the untreated flank organ, since the effect does not occur when DHT is applied in liposomes (Fig. 3). When DHT is applied directly to the flank organ the sebaceous structures increased in size, the effect of 20 and 40 μg DHT solved in acetone being greater than that obtained with the same amount of DHT, encapsulated in liposomes ($\alpha = 0.05$).

Mezei and Gulasekharam (3,4) held that the use of cutaneously applied liposomes would diminish systemic side-effects of encapsulated drugs. This effect, however, can only be partly affirmed by our experiments since although the stimulation of the untreated flank organ after androgen application occurs only after application of DHT in acetone, the drug in liposomes had a smaller effect than that applied in acetone, yet in the experiments of Mezei and Gulasekharam (3,4) a four times higher concentration of the tested substance was found when liposomes were used. Several factors might cause the discrepancy between their work and the present study:

1 Different steroids were used.
2 Mezei and Gulasekharam measured the concentration of the test substance in the skin, while we measured the biological effect.
3 Different species of animals were used (rabbits versus hamsters) and large differences in percutaneous absorption do occur between different species (1).
4 The schemes of application were different.

Since our results indicate that a liposome formulation shows a diminished systemic absorption in parallel with a reduced biological effect, we may conclude that DHT when applied in a liposome formulation shows no appealing advantages in comparison with more conventional delivery systems. Hence, this system is also not particularly convenient for the local administration of AHH-inducing agents to measure interindividual differences in AHH-inducibility in man, which have been suggested to play a role in susceptibility to chemical-induced neoplasia (see chapter 1).

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16

Absence of Induction of Aryl Hydrocarbon Hydroxylase after Topical Application of Corticosteroids

ABSTRACT

The effects of the topical application of beclomethasone dipropionate (a corticosteroid) on the aryl hydrocarbon hydroxylase (AHH) activity in lung and skin of male C3Hz mice were studied. To evaluate the results of the pre-treatment with beclomethasone dipropionate a comparison was made with the effects of the topical application of benz(a)anthracene (a polycyclic aromatic hydrocarbon and a known inducer of AHH-activity). AHH-activities were increased 2.5- to 5-fold in lung and about 20-fold in skin of mice after topical application of benz(a)anthracene. However, after pre-treatment with beclomethasone dipropionate no induction of AHH-activity could be observed, either in lung or in skin. For further testing the applicability of corticosteroids as AHH-inducing agents for assessment of interindividual differences in AHH-inducibility in man, two commercial corticosteroid preparations were applied to a small scalp region and AHH-activity measured in freshly isolated hair follicles. Both preparations were not able to increase AHH-activity significantly above the basal level. Hence, corticosteroids can not be applied for detection of interindividual differences in AHH-inducibility in man.

INTRODUCTION

Cigarette smoking has been shown to be epidemiologically related to lung cancer (10). The malignant transformation is believed to be initiated by exposure of the bronchial epithelium to aromatic polycyclic hydrocarbons such as benzo(a)pyrene (BP), which are

present in cigarette smoke and have been shown to be capable of inducing malignant tumors in laboratory animals. In most tissues polycyclic aromatic hydrocarbons (PAH) are metabolized to more hydrophilic products by the enzyme aryl hydrocarbon hydroxylase (AHH). During this process epoxides, probably more potent carcinogens than the hydrocarbons from which they are derived, are transiently formed. The diol-epoxides, very reactive intermediates that may be the ultimate carcinogenic hydrocarbons, are formed through the remetabolism of initially hydroxylated dihydrodiols (4). It has been demonstrated that individual differences in the rate of hydroxylation of PAH play an important role in determining the susceptibility to carcinogenesis by these agents in laboratory animals (9).

It has been described that topical application of corticosteroids induces AHH in skin (1). Since many dermatological patients, for example with psoriasis, may be exposed to these agents for periods extending over many decades, it is clearly of importance that the risk of developing cancer is properly assessed. Similarly, if the same phenomenon would occur in lung, patients with chronic nonspecific obstructive lung disease (CNSOLD) using topical corticosteroids may be at an increased risk of lung cancer from hydrocarbons with which they come in contact.

On the other hand, if corticosteroids can induce AHH-activity *in vivo* in man, short-lasting exposure to a limited scalp skin region can allow detection of interindividual differences in AHH-inducibility in human hair follicles. These differences have been suggested to play a role in susceptibility to chemical carcinogenesis (see e.g. 6 and ref. therein). We failed to observe any induction after topical application of beclomethasone dipropionate (BDP), neither in lung and skin of mice nor after application of two commercial corticosteroid preparations in human scalp hair follicles.

MATERIALS AND METHODS

Chemicals

BP was obtained from Aldrich Europe (Beerse, Belgium). [G-^{3}H]BP (specific activity 65 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, UK)

and was purified chromatographically before each experiment (silicagel t.l.c.; solvent system: toluene/ethanol (9 : 1)). Benz(a)anthracene (BA) was obtained from ICN K & K Labs (Plainview, N.Y., USA). BDP was from Glaxo, Greenford, UK. Betnelan-V® ointment (Glaxo) contained 0.1% betamethason-17-valerianate and Ultralan® cream (Schering, Berlin, FRG) consisted of 0.25% 6α-fluor-16α-methyl-1-dehydrocorticosteronetrimethylacetate and 0.25% 6α-fluor-16α-methyl-1-dehydrocorticosteronecapronate.

Animals

Male C3Hz mice (6-8 weeks) were obtained from the Central Animal Laboratory, Nijmegen, The Netherlands.

Administration of BA and BDP

For intratracheal instillation of BA and BDP, animals were anesthetized with chloralhydrate (4½% solution in water) and placed on special boards designed to hold their mouths open at a correct angle for instillation. After positioning of the animal, 100 µl of a BA- or BDP-solution (2 mg/ml in 0.9% sodium chloride/ethanol (9 : 1), supplemented with 5 µl of Tween 80) was delivered to the lung through the trachea. Controls were instilled intratracheally with the solvent solution.

Before topical application the mice were anesthetized with ether and shaved on the dorsal side with an electric clipper. BA and BDP (125 µg in 100 µl of acetone/arachis oil (9 : 1)) were applied twice daily to the dorsal side of the mice for a period of one week. Control mice were treated with solvent alone.

Enzyme assay

At 24 h after treatment animals were sacrificed by cervical dislocation. The lung or skin was excised and washed with 0.9% sodium chloride solution. The tissue was minced with the aid of scissors. In this crude preparation the AHH-activity was measured using two different methods (fluorometric measurement and radiochemical measurement). The fluorometric assays were carried out according to the method of Nebert and Gelboin (8). The radiochemical measurements were performed as described earlier (3). The only modification was that the labeled BP was diluted with unlabeled BP to a final concentration of 80 µM; 4 µCi was added to each incubation.

DNA was determined according to the method of Burton (2). Calf thymus DNA was used as a reference standard in the calculation of the DNA content. Enzyme activity was expressed as pmol BP metabolite/µg DNA/h.

Corticosteroid application in man

For the analysis of AHH-inducing potency in human hair follicles 5 daily doses of 0.5 g of Betnelan® ointment or Ultralan® cream were applied to a circled area of the scalp of about 3 cm. During this period the volunteers were asked not to wash their hair. Three persons were assessed for the AHH-inducing potential of both corticosteroid formulations. Volunteers were healthy non-smoking adults. For determination of basal AHH-activity 3 x 30 hair follicles were plucked from the untreated side of the scalp. In the treated area 3 x 30 hair follicles were collected for measurement of induced AHH-activity. AHH assays (in triplicate) and DNA-determinations in hair follicles were performed as described earlier (5; see also chapter 5). Enzyme activity was expressed as pmol phenolic BP metabolites/µg DNA/h.

The effect of the application of BPD and BA on the AHH-activity in mouse lung was studied. In the AHH assay BP was used as substrate for the enzyme, using two different methods (fluorometric and radiochemical). In the fluorometric assay the formation of 3-hydroxyBP was measured, whereas in the radiochemical method the total organo-soluble metabolites were assayed.

The effect of intratracheal instillation of BDP and BA on the BP metabolism is shown in Table 1 and Table 2. Both methods of AHH measurement gave similar results with regard to the formation of 3-hydroxyBP; the rate of 3-hydroxyBP formation was 1.2 pmol/ µg DNA/h in the control experiment (expressed as 100% in Table 1 and 2). Instillation of BA resulted in a considerable increase in AHH-activity; the induction ratios found in our experiments are in accordance with the results of Mitchell (7). It can also be concluded that the induction ratio is dose-dependent (for the doses used in the experiments). However, after instillation of BDP no induction, but rather a slight decrease in AHH-activity could be observed.

Table 1 Lung AHH-activity in C3Hz mice following intratracheal instillation of BA and BDP. Dose: 100 µg.

product formed	control	BDP-treated	BA-treated
fluorometric measurement:			
3-hydroxyBP	100 ± 12*	62 ± 7	282 ± 41
radiochemical measurement:			
3-hydroxyBP	100 ± 25*	56 ± 14	245 ± 29
7,8-dihydro-7,8-dihydroxyBP	49 ± 12	21 ± 6	108 ± 21
9,10-dihydro-9,10-dihydroxyBP	46 ± 11	25 ± 6	79 ± 15

**The amount of 3-hydroxyBP formed in the control experiment is expressed as 100% (mean ± standard deviation); each group contained eight animals.*

Table 2 Lung AHH-activity in C3Hz mice following intratracheal instillation of BA and BDP. Dose: 200 µg (twice that applied in Table 1).

product formed	control	BDP-treated	BA-treated
fluorometric measurement:			
3-hydroxyBP	100 ± 12*	67 ± 9	484 ± 62
radiochemical measurement:			
3-hydroxyBP	100 ± 21*	61 ± 12	483 ± 36
7,8-dihydro-7,8-dihydroxyBP	48 ± 11	20 ± 4	114 ± 20
9,10-dihydro-9,10-dihydroxyBP	46 ± 10	21 ± 4	76 ± 14

**The amount of 3-hydroxyBP formed in the control experiment is expressed as 100% (mean ± standard deviation); each group contained eight animals.*

Since we failed to observe induction of AHH-activity after intratracheal instillation of BDP (that was claimed to be an inducer of AHH in skin by Briggs and Briggs (1)), we decided to investigate the effect of topical application of a BDP solution on the skin of mice; in mouse skin the same pattern of BP metabolites was found as in mouse lung. The results of these experiments are shown in Table 3; after treatment with BA, the enzyme system in the skin was induced 20-fold, but after treatment with BDP no induction could be detected.

Table 3 Skin AHH-activity in C3Hz mice following topical application of BA and BDP (measured fluorometrically).

	3-hydroxyBP formed*
control	100 ± 23
BDP-treated	72 ± 18
BA-treated	1940 ± 150

**The amount of 3-hydroxyBP formed in the control experiment is expressed as 100% (mean ± standard deviation); each group consisted of eight animals.*

In the human experiments both commercial corticosteroid preparations did not elicit significant increase of AHH-activity in hair follicles (Table 4).

Table 4 AHH-activity in human hair follicles after 5 topical doses (0.5 g each) of Betnelan® V ointment or Ultralan® cream.

	3-hydroxyBP formed*
untreated	100 ± 24
Betnelan®	87 ± 20
untreated	100 ± 17
Ultralan®	109 ± 25

**The amount of fluorescence corresponding to 3-hydroxyBP formed in the hair follicles from the untreated scalp region is expressed as 100%.*

For each preparation three different volunteers were assessed.

DISCUSSION

In this study the effect of the local application of a corticosteroid on the AHH-activity in skin and lung of mice was studied; Briggs and Briggs (1) have suggested that humans receiving topical corticosteroid therapy might be at an increased risk of cancer from hydrocarbons with which they come in contact. This would indicate that situations, where patients are exposed to both corticosteroids and polycyclic aromatic hydrocarbons should be avoided. However, in our experiments we failed to demonstrate any induction of AHH-activity after corticosteroid pre-treatment: neither in lung nor in skin (the tissue Briggs and Briggs used in their experiments) induction was found, but rather a slight decrease in AHH-activity was demonstrated. The reason for this discrepancy could not be detected; since treatment with BA resulted in high induction ratios in lung and especially skin, this difference in results can not be attributed to the method of AHH

determination.

From our results for mouse tissues and for a human epithelial tissue, hair follicles, it can be concluded that there might be no significant difference in the risks of developing lung or skin cancer (due to exposure to polycyclic aromatic hydrocarbons) between control persons and patients treated topically with corticosteroids. However, this conclusion also implies that corticosteroids are not useful for the detection of interindividual differences in AHH-inducibility in man, which might be correlated to individual differences in susceptibility to chemical carcinogen-induced neoplasia (6).

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In Vivo Induction of Aryl Hydrocarbon Hydroxylase in Human Scalp Hair Follicles by Topical Application of a Commercial Coal Tar Preparation

ABSTRACT

Five low-dose applications of a commercial coal tar preparation on a small scalp skin region resulted in an induction of aryl hydrocarbon hydroxylase (AHH) activity in freshly isolated human hair follicles. Large but reproducible interindividual differences in AHH-inducibility could be detected. The method offers the opportunity to measure AHH-inducibility, which has been correlated to the risk of developing chemical-induced cancer, *in vivo* in normal epithelium, a cell-type highly relevant for chemical carcinogenesis. Smoking habits did not have any effect on AHH-activity in freshly isolated hair follicles. Therefore the method potentially permits the identification of persons with high and low genetically determined AHH-inducibility.

INTRODUCTION

Because of the high and increasing frequency of lung cancer and the low 5-year survival rate of this disease, efforts have been spent to find markers for early detection of lung cancer and/or identification of individuals at high risk. Although in animal model systems a relation between genetically regulated levels of aryl hydrocarbon hydroxylase (AHH) and susceptibility to chemical carcinogen-induced tumors has been found (2,17,18), reports from various laboratories investigating this relation in man have been contradictory (13,14,20,23). A likely source of this variation are the technical difficulties involved in achieving reproducible mitogen activation and subsequent AHH-induction in human peripheral

blood lymphocytes, the most widely used human cell-type in these studies (4,6,16). However, in a recent study many of the factors known to influence the mitogen activation step were controlled using a very rigid experimentation scheme (19). The data from these experiments showed a striking correlation between the presence of pulmonary carcinomas and high benz(a)anthracene induced AHH-levels in lymphocytes.

Unfortunately, for routine analysis this approach is not practical. It has been proposed that human hair follicles are a convenient biopsy tissue for assessing individual differences in carcinogen metabolism for various reasons (10,21; also p.92). Among these the epithelial origin of hair follicles and their easy availability are the most appealing ones. In this study we describe a method for the measurement of AHH-inducibility *in vivo* using human scalp hair follicles. The inducing agent is a commercial coal tar preparation (CT) employed in the treatment of skin diseases among which psoriasis and eczema. The method allows measurement of the genetically determined inducibility of a person. However, such approach can only be rational when smoking (which can be considered as one of the most obvious factors potentially influencing AHH-levels in the general population) does not affect AHH-levels in freshly isolated hair follicles. Therefore we have also investigated AHH-levels in hair follicles of a group of smokers and non-smokers.

MATERIALS AND METHODS

Chemicals

CT was obtained from the 'Onderlinge Pharmaceutische Groothandel', Velp, The Netherlands. Benzo(a)pyrene (BP) was purchased from Aldrich, Beerse, Belgium. Aceton, hexane and NaOH were of the Suprapure® grade (for fluorometry), Merck, Darmstadt, FRG. 3-Hydroxy-benzo(a)pyrene was obtained from the National Cancer Institute Chemical Repository (IIT Research Institute, Chicago, IL). A DNA fluorescence test combination based on the method of Hukkelhoven et al. (8) was purchased from Sanbio B.V., Nistelrode, The Netherlands (see also chapter 3).

Volunteers

Hair follicles were plucked with a forceps from healthy adult volunteers. Only hair follicles with visible bulb and sheath were used. To compare AHH-levels in smokers and non-smokers AHH-activity was determined in duplicate samples of 30 hair follicles. Smokers had a consumption between 5 and 30 cigarettes per day. The mean consumption was 14 cigarettes per day.

In vivo induction

For the induction of AHH-activity the following scheme was followed: a circled area of about 3 cm diameter at the scalp was marked with ink. To this area five applications of CT (0.5 ml) with an interval of 12 h were given. During this period the volunteers were asked not to wash their hair. The first application was at about 11 h p.m. As a result the last one was two days later at the same time. In the morning of the next day the volunteers were asked to wash their hair thoroughly to remove exogenously absorbed CT. After this hair follicles were plucked. For determination of basal AHH-activity 3 x 30 hair follicles were plucked from the other side of the scalp. In the marked area 6 x 30 hair follicles were plucked: 3 x 30 for determination of induced AHH-activity and 3 x 30 for a tissue blank.

Measurement of AHH-activity in hair follicles

For measurement of AHH-activity the method of Hukkelhoven et al. (9) was basically used. In short, 30 hair follicles in a 1 ml Eppendorf tube were incubated in 200 µl of a solution of 50 mM Tris pH 8.5, 2 mM $MgCl_2$ and 80 µM BP at 37°C during 1 h. The hair follicles of the tissue blank did not contain BP in the medium. The reaction was stopped with 200 µl -20°C aceton. Organic solvent-soluble metabolites were extracted with 800 µl hexane and phenolic BP-metabolites with 1 ml of 1 N NaOH added to the isolated hexane phase. Fluorescence in the alkaline phase was determined in a Perkin-Elmer 650-40 fluorometer at an excitation wavelength of 396 nm and an emission wavelength of 522 nm. 3-OH-BP was used for the preparation of a calibration curve and quinine HBr in 0.1 N H_2SO_4 was employed for calibration of the spectrofluorometer.

Measurement of DNA in hair follicles

After enzyme determination the hair follicles were transferred to a new Eppendorf tube and 1 ml of distilled water was added. After this DNA was measured according to the mithramycin method of Hukkelhoven et al. (8) using a commercially available DNA fluorescence test combination (see also chapter 3). Enzyme activity was expressed as pmol phenolic BP metabolites/µg DNA/h. Induction ratios were calculated as the induced AHH-activity divided by the basal AHH-activity.

RESULTS

Influence of coal tar on tissue blanks

Even after extensive washing of the hair after the last coal tar application, some coal tar remains associated with the hair. When hair follicles plucked from the coal tar region are incubated without BP, a relatively high background fluorescence is obtained. This background can be decreased by removing the shaft of the hair almost completely, i.e. by cutting the hairs right above the sheath of the hair follicle. For calculation of induced AHH-activity the fluorescence of these tissue blanks is subtracted from

the fluorescence obtained when hair follicles are incubated togethet with BP. For calculation of basal activities, only normal blanks (medium and BP) were subtracted from the fluorescence obtained when untreated hair follicles were incubated with BP. No fluorescence was extracted from hair follicles from the untreated skin.

Kinetics of AHH-induction

To determine how many applications of CT are necessary for maximal AHH-induction, one volunteer was subjected to several application schemes. One series of experiments was conducted using diluted CT (CT : alcohol = 1 : 1) which was applied in an amount of 0.5 ml during 3, 5 and 10 treatments. Another series involved the application of 0.5 ml undiluted CT during 1, 2, 3 and 5 treatments. Between each treatment a time period of 12 h was chosen. The results (Fig. 1) clearly show that with the diluted CT submaximal induction is achieved even after 10 applications. On the other hand with undiluted CT maximal induced activity is already reached after 3 applications. As a routine procedure, however, 5 applications of 0.5 ml undiluted CT have been chosen.

It was further investigated whether the effect of CT on AHH-activity was limited to the treated scalp region. For this purpose AHH-activity outside the marked area was measured in three persons before and after application of CT. The results showed similar enzyme activities before and after treatment indicating that the effect of CT on AHH-activity is restricted to the treated skin surface.

Individual variation in AHH-inducibility

Ten volunteers were measured for their AHH-inducibility. It was found that inducibility figures varied greatly. The highest value was 4.9 and the lowest 1.1. Mean inducibility was 2.8 ± 1.2. Five persons were reassessed after 10 weeks. Inducibility ratios were reproducible, with the largest deviation being 34% and the mean deviation 22% (Table 1).

Since in the relatively small application area 180 hair follicles were plucked, in most volunteers a more or less bald spot

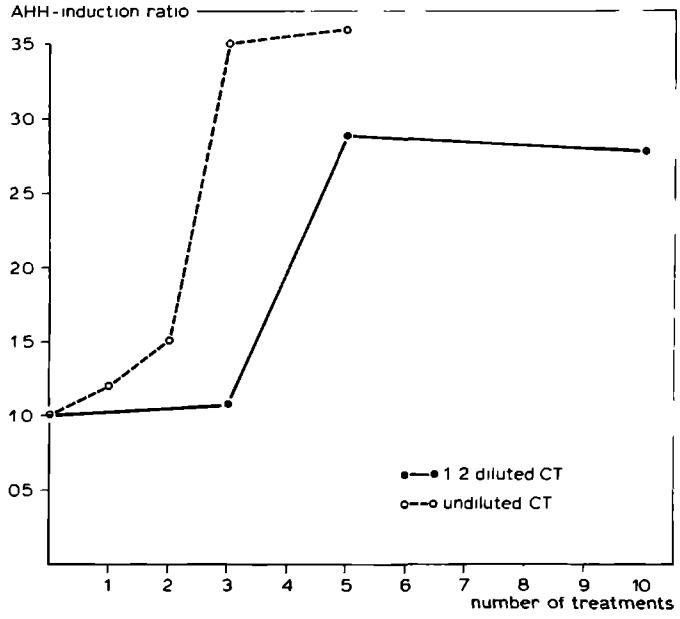

Fig. 1 Kinetics of AHH-induction after various applications of undiluted and diluted CT (CT : ethanol = 1 : 1). Between each treatment a time interval of 12 h was chosen. All measurements were performed with the same volunteer.

Table 1 Reproducibility of *in vivo* AHH-inducibility assay

volunteer	first assay	second assay	deviation*
I	1.72	2.31	34
II	3.11	3.73	20
III	4.87	4.12	18
IV	3.74	3.20	17
V	1.73	2.09	21
mean	3.03	3.09	22

**expressed as* $\{(\frac{highest\ value}{lowest\ value} \times 100) - 100\}\%$

Five different persons were assessed two times with an interval of 8-12 weeks. Figures represent inducibility ratios.

on the scalp resulted. However, after 18 weeks this area was again fully grown with new hair.

Effects of smoking habits on AHH-activity

In Fig. 2 the AHH-activities of the hair follicles of smokers and non-smokers are shown. Table 2 summarizes the data obtained from both groups. By means of the Shapiro-Wilk test, it was found that the results from the non-smokers did not correspond to a normal distribution. However, a normal distribution in both groups could be obtained by a logarithmic transformation of the enzyme activities. 95% Confidence limits were calculated for the difference of the mean transformed data. After retransformation of the confidence limits the ratio of the medians of the non-smokers and the smokers groups could be obtained (the median is the enzyme activity where at both sides 50% of the determinations are found). The 95% confidence limits of this ratio (non-smokers vs. smokers) were 0.94 and 1.38 which indicates no significant difference between both groups.

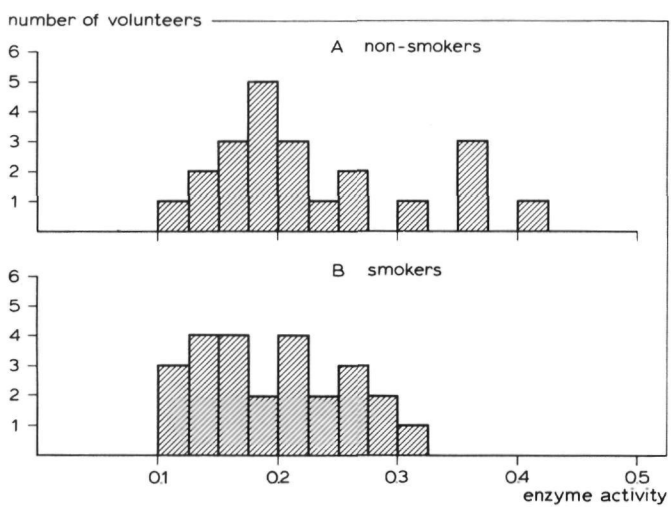

Fig. 2 Distribution of AHH-activities in the group of smokers (A) and non-smokers (B). Enzyme activity is expressed as pmol phenolic metabolites/µg DNA/h.

Table 2 Statistical data obtained from smokers and non-smokers

	non-smokers	smokers
number	22	25
mean enzyme activity*	0.228	0.197
S.D.	0.081	0.059
median	0.204	0.197

**expressed as pmol phenolic metabolites/μg DNA/h.*

DISCUSSION

The prognosis for patients with bronchogenic carcinoma is at only 5 to 15% overall survival for five years postdiagnosis even with therapeutic intervention (7). Therefore prevention should have priority in the control of the disease. This can be achieved by determining the individuals at greater risk to the development of lung cancer (5) and/or removal of those agents for which clear evidence exists that they are closely associated with the aetiology of the disease. However, social and economic factors have up till now prevented a fulfilment of the latter alternative.

Detection of individuals at risk has received a lot of scientific attention since Kellermann's observations linking susceptibility to chemical carcinogenesis with high inducibility of AHH in humans (14). Although these results could not be reproduced by some other laboratories the topic has received new attention by the development of techniques making the human hair follicle available as an epithelial biopsy tissue (for a review see 22) as well as by a convincing study of Kouri et al. (19), who found a striking relationship betweeen benz(a)anthracene induced AHH-levels and the presence of bronchogenic carcinoma.

In this study we have presented a method for measurement of coal tar induced AHH-levels in freshly isolated human hair follicles. Human hair follicles are composed of epithelial cells, the same cell-type which makes up most human malignancies. It has to

be realised that coal tar and some of its constituents are carcinogenic in animal bioassays (15). However, in psoriatic patients who are often treated with large doses of coal tar, the incidence of skin cancer does not appear higher than in the rest of the population (12). Percutaneous absorption and entrance in the systemic circulation of some organic compounds of CT must also be considered as a possible event. Epidemiological studies on the frequency of other types than skin cancer in psoriasis patients are now being conducted (see 25). However, the dose applied for the AHH-inducibility assay is so low and infrequent compared to that used in the treatment of psoriasis that the risks for volunteers are negligible. Moreover, percutaneous absorption in psoriatic skin may even be greater than in normal skin (3).

The technique presented in this study provides the opportunity to measure AHH-inducibility *in vivo* in man in a cell-type highly relevant for chemical carcinogenesis. In earlier studies induction of microsomal monooxygenase activity in human placentas (24) and in human alveolar macrophages (1) has been described. It is however for the first time that AHH-inducibility *in vivo* in a human epithelial biopsy tissue suitable for population studies has been shown unequivocally. It has already been shown that BP-metabolism of hair follicle cells is largely comparable to that in the target tissue for polycyclic aromatic hydrocarbon-induced carcinogenesis, the tracheo-bronchial epithelium (11; see also chapter 10). In this study we also found that smoking habits do not influence AHH-activity in hair follicles, so the AHH-inducibility ratio will largely reflect genetic traits. Therefore the technique can be used to further investigate whether AHH-inducibility is correlated to the risk of developing bronchogenic carcinoma. When this relation has been established, the development of a more sensitive technique of AHH-determination (employing smaller quantities of hair follicles) will further facilitate the identification of high risk groups.

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PART 6

SUMMARY AND GENERAL DISCUSSION

18

Summary and General Discussion

As has been discussed in Part 1 of this thesis, both environmental factors (including dietary and smoking habits and exposure to naturally occurring and man-made environmental carcinogens) and genetic factors seem to play an essential role in the etiology of cancer. Therefore prevention of the disease can be approached both by elimination of environmental risk factors as well as by identification of individuals genetically predisposed to cancer. The first alternative is in general difficult to achieve both for socio-economic reasons and since for most cancers the really causative agents are not (yet) identified. However, a more direct causal relation between chemicals and human cancer has been established for certain occupationally related cancers and for lung cancer in cigarette smokers. For this reason and because bronchogenic carcinoma is the most fatal neoplastic disease in the Western world, an effective anti-smoking campaign is undoubtedly essential for public health.

The other determinant of human cancer, genetically based susceptibility, has been the subject of much research during the last ten years. The underlying aim of these studies has been the idea that the ability to predict the relative individual susceptibility to specific carcinogens would enable to reduce the exposure to carcinogens for the individual concerned. The eventual possibility to identify high risk populations will, of course, not obviate the need to remove notorious hazards from our environment. (It should be noted that some authors prefer the term 'predisposing host factors' instead of 'predisposing genetic factors'. Host factors include genetic factors and acquired conditions which can sometimes be associated with increased cancer risk, such as infectious and inflammatory conditions).

From the concept of metabolic activation of most carcinogens and from animal studies indicating a role of activating enzyme systems in chemical carcinogenesis, it has been speculated that persons at high risk have an increased activation-to-deactivation ratio of chemical carcinogens. However, it has also been realized that other parameters might be important such as DNA-binding, immune surveillance and tumor promotion. Another major problem in assessment of oncogenic susceptibility has been the lack of a proper human biopsy tissue. In view of the prevalence of carcinomas and tissue specific differences in carcinogen metabolism it has been recognized that such a tissue should be of epithelial origin. In this thesis the introduction of human hair follicles for assessment of individual differences in benzo(a)pyrene metabolism, is motivated.

Part 2 deals with analytical procedures and techniques that had to be developed in order to allow application of this biopsy tissue. First, it was realized that measurement of certain parameters of carcinogen metabolism, such as induction of carcinogen metabolizing enzymes and binding of reactive intermediates to DNA, could not be performed in freshly isolated hair follicles. Therefore, in Chapter 2 a technique is described enabling the culture of epithelial cells on a basement membrane-like substrate, the bovine eye lens capsule. This technique has permitted the development of a routine method for culturing human hair follicle keratinocytes. I feel that studies with these normal, truly epithelial human cells may provide a very important link between clinical observations and *in vivo* animal studies.

For measurement of biochemical parameters in hair follicles a reference variable has to be employed which can relate e.g. enzymatic activity to the number of cells present. Since protein is not convenient for this purpose, due to the large contribution of dead keratinized cells, a sensitive DNA-assay has been developed which even permits measurement of DNA in fractions of hair follicles (Chapter 3). Both techniques - the culture system and the DNA-assay - can now be applied in other laboratories and research fields, since they have been made commercially available. Since the use of hair follicles represents a new item in toxicology,

further methods had to be developed for measurement of parameters of carcinogen metabolism. Because we aim at an application in large scale screening programmes, it was realized that assays for carcinogen metabolizing enzymes should not be hampered by laborious techniques or practical inconvenience. Therefore the use of radioactive substrates and time-consuming extraction/isolation procedures, extensively employed in existing methods, had to be avoided.

BP-metabolites can be expected to exhibit large intrinsic fluorescence intensity. Therefore efforts were made to develop enzyme assays based on fluorescence measurement of the metabolite involved. To further facilitate these determinations two technical devices for enhancement of sensitivity in fluorescence spectrometry were tested. Both methods - a cell-holder containing two concave mirrors and an aluminium-coated normal fluorescence cell - resulted in an increase in sensitivity by a factor 2.6-2.7 (Chapter 4).

With the aid of this system assays for three different enzymes involved in benzo(a)pyrene (BP) metabolism were developed:

1 Aryl hydrocarbon hydroxylase (AHH), one of the mixed-function oxidases, represents the key enzymologic interface between humans and foreign chemicals among which polycyclic aromatic hydrocarbons (PAH). AHH-activity can be increased on exposure to various chemicals and drugs in most animal and human tissues. Differences in AHH-inducibility in various strains of mice have been correlated to their susceptibility to carcinogenesis by some PAH. In men, conflicting reports on the role of AHH-inducibility in bronchogenic carcinoma have appeared. Most of these studies have employed human lymphocytes. The present possibility of measuring AHH-activity in freshly isolated hair follicles as well as AHH-inducibility in cultured hair follicle keratinocytes (Chapter 5), can possibly resolve the controversy on this important subject.

2 Another enzyme for which an assay especially suitable for use of hair follicles has been developed is epoxide hydrolase (EH; Chapter 6). This enzyme has a dual role in toxification and detoxification of PAH. On the one hand it inactivates the electrophilically primary epoxides, at the other hand it produces

the substrates (dihydrodiols) for the AHH-mediated oxygenation to the proposed ultimate carcinogens of PAH, the diol-epoxides.

3 Glutathione-transferase is generally considered to act detoxifying, since it inactivates arene oxides and, as has been shown recently, also diol-epoxides. The sensitivity of the assay and the high intrinsic fluorescence of the conjugate permits the enzyme to be measured in only one freshly isolated hair follicle (Chapter 7).

In recent years the delicate balance between enzymes which potentiate and those which detoxify reactive intermediates rather than one specific enzyme activity, is being increasingly appreciated. Therefore we developed an HPLC-method which enables the determination of all organic solvent-soluble [^{3}H]BP metabolites in freshly isolated hair follicles or in cultured hair follicle keratinocytes (Chapter 8). In the latter case it is also possible to measure inducibility of BP-metabolites after pre-exposure to PAH or other xenobiotics.

Chemical mutagenesis and carcinogenesis are believed to find their onset in the covalent binding of reactive intermediates to DNA. We have developed a method for the isolation of [^{3}H]BP-modified DNA from hair follicle keratinocytes. After enzymic degradation of the DNA, fractionation of the resulting mixture by Sephadex LH-20 column chromatography and subsequent HPLC-analysis of the adducts, the main covalent interaction was shown to result from the binding of the C-10 position of trans-7,8-dihydrodiol-9,10-epoxyBP to the N2 exocyclic position of guanine (Chapter 9). Since a rather good correlation exists between 'true binding' as represented by identified adducts after HPLC-analysis and 'total binding', as calculated from the specific activity of the isolated DNA, the latter parameter can serve as an alternative for the rather time-consuming determination of 'true binding'.

In Part 3 of this thesis evidence is presented that human hair follicles can be used as indicator organs for individual differences in carcinogen metabolism. In Chapter 10 BP-metabolism in human hair follicles is compared with that in the most important target-tissue for PAH-induced neoplasia, the tracheo-bronchial epithelium.

It was concluded that freshly isolated hair follicles, cultured hair follicle keratinocytes and cells cultured from human bronchial epithelium yielded quantitatively comparable amounts of the most important organic-soluble BP metabolites: early eluting compounds (probably tetrols), 9,10-dihydrodiol, 7,8-dihydrodiol, quinones and phenols. AHH-activity in both cultured cell-types was highly inducible after pre-exposure to benz(a)anthracene. The metabolic pattern was also compared to that of lymphocytes and monocytes, which have been used frequently in population studies and with data from other types of epithelial cells. It can be concluded that BP-metabolism in freshly isolated hair follicles and in cultured hair follicle keratinocytes is comparable to that in cultured epithelial cells from organs for which evidence of a role of chemical carcinogens in the development of cancer of that organ has been provided.

In Chapter 11 it was investigated whether genetic factors can account for - at least some - of the interindividual differences in carcinogen metabolism. For this purpose basal levels of BP-conversion to dihydrodiols in hair follicles of monozygotic twins, dizygotic twins and pairs of unrelated individuals were analyzed. Intra-twin differences in BP-metabolism for monozygotic twins were smaller than for dizygotic twins and intra-pair diffrences for dizygotic twins were smaller than for pairs of unrelated individuals. These results clearly suggest that individual differences in BP-metabolism towards dihydrodiols, among which the proximate carcinogen 7,8-dihydrodiolBP, are partly genetically determined. Chapter 12 deals with the possible relation between formation of phenolic and dihydrodiol metabolites of BP in freshly isolated hair follicles. No correlation between the amount of these types of metabolites formed, could be detected. Thus, if the concentration of phenolic or dihydrodiol metabolites plays an important role in the biological effect of PAH, measurement of these two types of metabolites can not be interchanged in assessing an individual's susceptibility to the carcinogenic action of BP.

In Part 4 some aspects of BP-metabolism in human and murine tissues are compared to each other. The rationale for this is, that when roughly the same metabolic characteristics are apparent

in both species, the animal can function as a test system for the elucidation of the factors responsible for the oncogenic process. We chose the inbred strain C3Hz which is frequently used in chemical carcinogenesis studies. As described in Chapter 14 and 15, there is a consequently appearing difference between human and murine test systems. Whereas pre-exposure to benz(a)anthracene (BA) resulted in a large increase in AHH-activity (reflected by the formation of phenolic metabolites) in both species, this treatment had no influence on the amount of dihydrodiol BP-metabolites in culture systems of human origin. On the contrary, dihydrodiol metabolites of BP had largely increased in murine test systems upon pre-exposure to PAH. We think that this important difference in response to pre-treatment with PAH, precludes direct extrapolation of data obtained in experimental animals to the human situation. We are presently investigating the relation between susceptibility to BP-carcinogenesis and various metabolic parameters in three different inbred strains of mice: C57Bl/6, DBA/2 and C3Hz. Analysis of a large number of biochemical factors (basal and induced levels of AHH and EH, glutathione-transferase, DNA-binding, ornithine decarboxylase induction as a measure of tumor promotion) can possibly shed light on the large differences in genetically determined susceptibility to BP-carcinogenesis that exist in these strains. When the important factors can be identified, the human hair follicle comprises an ideal organ to investigate whether the same relations are valid in men. This can be accomplished by analyzing lung and laryngeal cancer patients, persons with other forms of cancer and healthy individuals. Although there is still controversy about the role of AHH-inducibility in chemical-induced cancer in man, the most carefully designed experiments in recent years show that: (1) individuals exhibit variation in AHH-inducibility which is, at least partly, under genetic control and (2) the mean inducibility of patients with lung or laryngeal cancer is higher than that of the normal population. Anticipating on the results from the described animal experiments, we are therefore now already measuring BA-induced AHH-levels in cultured hair follicle keratinocytes from lung and laryngeal cancer patients.

In Part 5 investigations are described in which three methods for the induction of AHH-activity in humans *in vivo* are tested:

1 Since induction of AHH-activity *in vivo* has to be restricted to a small area of the scalp region from which the hair follicles are obtained, we tested the hypothesis whether liposomes (phospholipid bilayer vesicles) might be an effective application form for AHH-inducing substances. As a model system for studying the local and systemic effects of drugs, we applied liposome-encapsulated steroids to sebaceous structures in hamsters. Under the experimental conditions used, unequivocal advantages of the liposome system over application in acetone could not be demonstrated (Chapter 15). Therefore, the liposome system with encapsulated AHH-inducing substances is not markedly more efficient for determining interindividual differences in AHH-inducibility than the classical application in organic solvents.

2 It has been described that topical application of corticosteroids induces AHH-activity in skin of mice. To test the applicability of corticosteroids as AHH-inducing agents for assessment of individual differences in AHH-inducibility, these compounds were applied to a small region of the human scalp skin and to mouse skin (Chapter 16). In neither of the species an elevation of AHH-activity could be detected. It was concluded that corticosteroids are not useful for measurement of genetically determined AHH-inducibility in man.

3 In Chapter 17, however, evidence is presented that a commercial coal tar preparation, widely used for the treatment of skin diseases, can be employed for this purpose. In this study it is also shown that the level of AHH-activity in scalp hair follicles is not influenced by smoking habits. This of course is a prerequisite for ascribing measured differences in AHH-inducibility to genetic factors. We are presently investigating whether *in vitro* AHH-inducibility ratios obtained with cultured hair follicle keratinocytes are a good reflection of *in vivo* AHH-inducibility as measured in freshly isolated hair follicles.

In conclusion, although experimental animal and human studies strongly point to a relationship between carcinogen metabolism and cancer susceptibility, the fundamental parameters are not yet known. In addition the 'classical' test for human AHH-inducibility using cultured lymphocytes is at best a difficult one, even for research purposes. In the work described in this thesis we have developed a number of procedures employing human hair follicles from which a clinically useful procedure can be generated and evidence is presented that these organs are a relevant biopsy tissue for this purpose. The studies which are at the moment being performed in our laboratory can possibly solve the question which tests on hair follicles are important to identify highly susceptible individuals.

Toelichting voor de Geïnteresseerde Leek

Met deze toelichting zal ik proberen U als niet biochemisch geschoolde lezer een indruk te geven van de inhoud van dit proefschrift. Uitgangspunt voor mijn onderzoek was dat zowel omgevingsfactoren als erfelijke eigenschappen een grote rol spelen bij het ontstaan van kanker bij de mens. Bij omgevingsfactoren moeten we niet alleen denken aan verontreinigingen in lucht, water en bodem, maar evenzeer aan onze eet- en drinkgewoonten, roken en blootstelling aan natuurlijke en kunstmatige straling. Hoewel slechts enkele zeer zeldzame vormen van kanker duidelijk erfelijk zijn bepaald, blijkt erfelijke aanleg bij veel kankergevallen tóch een rol te spelen. Kanker kan waarschijnlijk pas ontstaan wanneer het betreffende individu een erfelijke aanleg voor kanker heeft én er bovendien blootstelling aan bovengenoemde omgevingsfactoren (meestal kankerverwekkende stoffen) heeft plaatsgevonden. Dit wordt geïllustreerd door het eenvoudige gegeven dat weliswaar bijna alle longkankerpatiënten rokers zijn (geweest), maar niet alle zware rokers longkanker krijgen. Rokers die longkanker ontwikkelen blijken meestal ook een erfelijke aanleg voor deze ziekte te hebben.

Roken

Hoe kun je iets veranderen aan de nog steeds toenemende sterfte aan longkanker? De beste oplossing is niet (meer) roken, dat wil zeggen het vermijden van de bij longkanker belangrijke omgevingsfactor. Als aanvulling hierop is het zinvol aandacht te schenken aan de tweede risicofactor: de erfelijke aanleg voor longkanker. Aan deze aanleg zelf kan voorshands niets worden veranderd. Wanneer we echter kunnen vaststellen welke mensen als gevolg van hun aanleg een verhoogd risico lopen, dan kunnen deze personen beter worden gecontroleerd en misschien beter worden gemotiveerd met roken te stoppen. Om deze reden was het doel van ons onderzoek methoden te ontwikkelen waarmee deze aanleg kan worden vastgesteld. Daarnaast kan kennis van de erfelijke aspecten van longkanker van nut zijn voor het begrijpen van het ontstaan van allerlei andere vormen van kanker.

AKH

Wat moet je je nu voorstellen bij deze erfelijke factor? Onderzoek bij proefdieren levert talrijke aanwijzingen dat de activiteit van een bepaald enzymsysteem hierbij een belangrijke rol speelt (een enzym is een eiwit dat verantwoordelijk is voor het verloop van sommige scheikundige reacties in de cel). Het enzym reageert met allerlei gevaarlijke stoffen die het lichaam binnenkomen, bijvoorbeeld in de vorm van sigaretterook. Het gevolg van deze reacties is, dat er stoffen ontstaan die sterk kankerverwekkend zijn. Hoe actiever dit enzymsysteem is, des te meer kankerverwekkende produkten er ontstaan en des te groter de kans op kanker wordt. Het enzym wordt meestal afgekort als AKH. Deze AKH-activiteit bepaalt dus in feite de erfelijke aanleg voor longkanker, althans bij muizen. Of het bij de mens ook zo is, moet nog blijken. Als dit wel het geval mocht zijn, betekent het, dat er mensen zijn met een aangeboren verhoogde enzymactiviteit. Door deze eigenschap heeft men een verhoogd risico voor longkanker.

In dit proefschrift wordt een methode uitgewerkt om deze enzymactiviteit in menselijke haarwortels te meten. Er zijn natuurlijk een aantal redenen om juist dít materiaal te nemen. De belangrijkste reden is wel dat haarwortels uit een type cellen bestaan die, wat betreft vorm en oorsprong, veel lijken op de cellen die bij longkanker zijn ontspoord. Een groot praktisch voordeel is dat bij de meeste mensen haarwortels vrijwel pijnloos kunnen worden geplukt.

Andere biochemische processen

De manier waarop chemische stoffen kanker kunnen veroorzaken, is echter zeer ingewikkeld. Niet alleen het hierboven beschreven enzym AKH speelt een rol, maar ook andere biochemische processen kunnen belangrijk zijn. Let wel: in welke mate de verschillende omzettingsprocessen van kankerverwekkende stoffen bijdragen tot het ontstaan van kanker is nog niet bekend. Dit moet nog blijken uit dierproeven en experimenten met haarwortels van zowel longkankerpatiënten als, ter vergelijking, patiënten met andere ziekten. In dit proefschrift zijn verschillende hoofdstukken gewijd aan de ontwikkeling van methoden om deze processen te meten. Voor sommige metingen kunnen cellen van 'vers geplukte' haarwortels

worden gebruikt, andere metingen maken gebruik van *gekweekte* haarwortelcellen. Dit zijn cellen die buiten het lichaam een lange tijd in leven worden gehouden en zich daar ook kunnen vermenigvuldigen. Biochemische processen (zoals enzymactiviteiten) worden altijd gemeten per miljoen cellen. Daarom werd een methode ontwikkeld om het aantal cellen in een kweek te bepalen. Zowel deze techniek als de methode voor het kweken van cellen zijn thans commercieel beschikbaar.

Luchtpijpbekleding

We hebben nu de beschikking over methoden voor het meten van de omzetting van kankerverwekkende stoffen in haarwortels. We weten echter nog niet of haarwortels ook geschikt zijn voor ons hoofddoel: de bepaling van erfelijke factoren bij longkanker. Een eerste voorwaarde hiervoor is natuurlijk dat de processen, die wij in haarwortels kunnen meten, vergelijkbaar zijn met die in het celtype dat bij longkanker is aangetast. Daarom is in het volgende deel van mijn proefschrift de omzetting van een bekende kankerverwekkende stof in haarwortels vergeleken met die in cellen van de luchtpijpbekleding. Het is gebleken dat de omzetting in beide celtypen vrijwel op dezelfde manier verloopt. Gegevens verkregen door metingen aan haarwortels geven dus een goede indruk van wat er in dit opzicht in de long gebeurt.

Een andere voorwaarde is dat de omzetting van kankerverwekkende stoffen in haarwortels inderdaad erfelijk is bepaald. Dit kon worden onderzocht met behulp van een aantal een-eiïge tweelingen, twee-eiïge tweelingen en met combinaties van twee niet verwante personen. Gebleken is dat de verschillen tussen de paren het kleinst waren bij een-eiïge tweelingen. Aangezien een-eiïge tweelingen erfelijk identiek zijn, is dit een goede aanwijzing dat de biochemische omzettingen voor een groot deel erfelijk zijn bepaald en in mindere mate worden beïnvloed door min of meer toevallige gebeurtenissen zoals dieet, beroep, medicijngebruik etc. Haarwortels zijn dus inderdaad 'orgaantjes' waarmee erfelijke verschillen in de omzetting van kankerverwekkende stoffen kunnen worden gemeten!

Kankerpatienten

Of, en zo ja in welke mâte deze verschillen ook uiteindelijk beslissend zijn voor het ontstaan van kanker wordt momenteel in ons laboratorium onderzocht. Daarbij spelen experimenten met behulp van ingeteelde muizenstammen met kleine, bekende verschillen in erfelijke eigenschappen, een rol. Door in deze dieren experimenteel tumoren op te wekken, kan worden onderzocht door welke factoren de ene stam gevoeliger is voor kanker dan de andere. Vervolgens kan door meting in haarwortels van kankerpatiënten en van gezonde personen worden bepaald of deze factoren ook bij de mens het ontstaan van kanker beïnvloeden. Vooruitlopend op de resultaten zijn we nu reeds bij kankerpatiënten en bij gezonde personen het AKH-enzym aan het meten, omdat er veel aanwijzingen zijn dat dit de belangrijkste rol speelt.

Conclusie

Samenvattend kan worden gezegd dat in het hier beschreven onderzoek is aangetoond dat de menselijke haarwortel potentieel geschikt is voor het meten van verschillen in gevoeligheid voor kankerverwekkende stoffen. Bovendien hebben wij een aantal methoden ontwikkeld waarmee deze verschillen kunnen worden gemeten. Het nog lopende onderzoek kan ons een antwoord geven op de vraag in welke mate de verschillende factoren de gevoeligheid voor kanker bepalen. Overigens valt het te hopen dat mensen zonder erfelijke aanleg voor longkanker door een dergelijk onderzoek niet worden aangespoord juist wél te roken. Want roken levert naast longkanker nog veel meer risico's op. Maar dat is weer een heel ander verhaal.

Curriculum Vitae

Math W.A.C. Hukkelhoven was born the 24th of November 1953 at Echt. After he graduated from the 'Scholengemeenschap Sint Michiel' at Geleen, he started his Biology studies in 1972 at the University of Nijmegen where he obtained his Bachelor's Degree (B4) in 1975.

His M. Sc. included the following subjects: Plant Physiology (Prof. Dr. H.F. Linskens) at the Unilever Research Laboratory at Duiven, Animal Physiology (Prof. Dr. A.P. van Overbeeke) carried out at the Hospital Sint Annadal Maastricht and Vegetation Science (Prof. Dr. V. Westhoff) for which research was done in Egypt in the framework of an international ecological project; his major subject was Biochemistry (Prof. Dr. H. Bloemendal) carried out at the Department of Biochemistry University of Nijmegen. He recieved his Master's Degree with honors in March 1979.

From March 1979 until February 1984 he was appointed at the Research Unit for Cellular Differentiation and Transformation, University of Nijmegen (Head: Dr. A.J.M. Vermorken); during 3½ years as Research Fellow of the Netherlands Cancer Society, thereafter employed by the University of Nijmegen as a scientist financed by the Netherlands Cancer Society. The research carried out during this period is described in this thesis. Two of the techniques which were developed in the framework of the research have been made suitable for commercial exploitation which is performed by Sanbio B.V. at Nistelrode (Chapter 2 and 3).

In November 1979 he visited the Departments of Pharmacology and Dermatology of the University of Newcastle as part of a technology-transfer programme of the International Union against Cancer. In September 1981 he attended a course on DNA repair at Brighton, sponsored by the European Community. He presented papers on international congresses in Halle (GDR), Athens, Helsinki and Stockholm.

Since March 1979 he is an Associate Editor of Ophthalmic Literature. In 1981 he followed a post-academic course on Science Journalism.

He joined Organon International B.V. at Oss in February 1984. His responsibilities include the handling of scientific questions of health authorities and the compilation and up-dating of the scientific information for the marketing department. He is further an advisor of the Research Unit for Cellular Differentiation and Transformation University of Nijmegen.

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1 F.C.S. Ramaeckers, M.W.A.C. Hukkelhoven, A. Groeneveld and H. Bloemendal. Cytoskeletal structures in cultured bovine lens cells and their role in lens cell elongation. Ophthalmic. Res. 11: 283, 1979.

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Curriculum Vitae

Math W.A.C. Hukkelhoven werd geboren op 24 november 1953 te Echt. Na het behalen van het Atheneum-B diploma aan de Scholengemeenschap Sint Michiel te Geleen begon hij in 1972 met de biologiestudie aan de Katholieke Universiteit te Nijmegen waar hij in 1975 het kandidaatsexamen B4 aflegde.

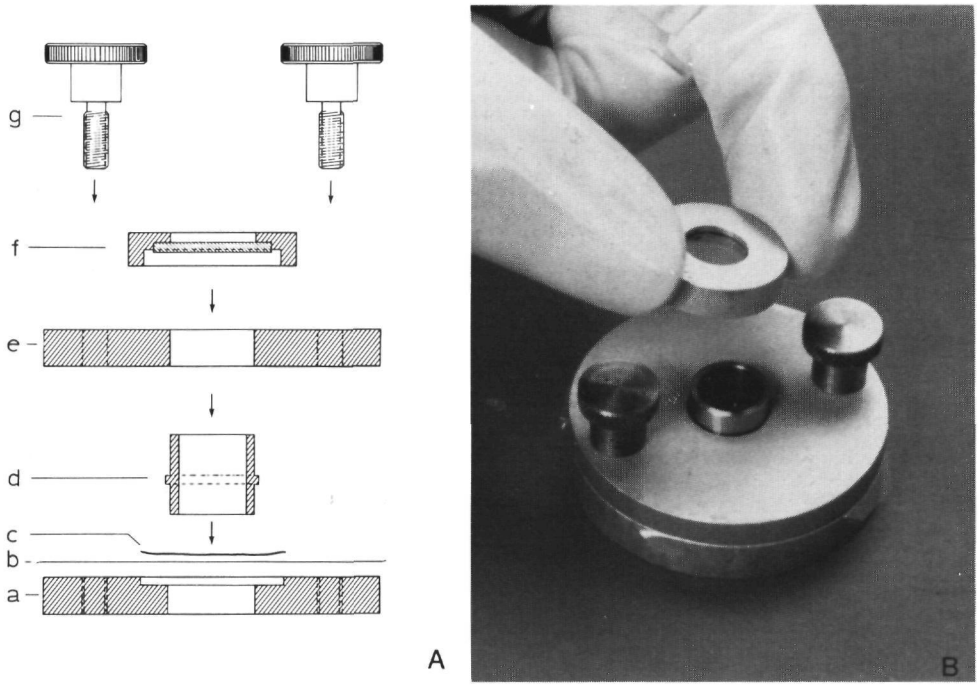
De doctoraalfase omvatte de volgende onderdelen: Plantenfysiologie (Prof. Dr. H.F. Linskens) verricht op het Unilever Research Laboratorium te Duiven, Dierfysiologie (Prof. Dr. A.P. van Overbeeke) uitgevoerd in het Ziekenhuis Sint Annadal te Maastricht en Geobotanie (Prof. Dr. V. Westhoff) waarvoor onderzoek werd gedaan in Egypte in het kader van een internationaal ecologisch project; het hoofdvak Biochemie (Prof. Dr. H. Bloemendal) werd verricht op het Biochemisch Laboratorium van de Katholieke Universiteit Nijmegen. Het doctoraalexamen werd in maart 1979 cum laude afgelegd.

Van 1 maart 1979 tot 1 februari 1984 was hij verbonden aan de Werkgroep voor Cellulaire Differentiatie en Transformatie (Hoofd: Dr. A. Vermorken) van de Nijmeegse Universiteit; de eerste 3½ jaar als Research Fellow in dienst van het Koningin Wilhelmina Fonds (KWF), later als wetenschappelijk medewerker in dienst van de K.U. Nijmegen gefinancierd middels een project van het KWF. Het in deze periode verrichte onderzoek is beschreven in deze dissertatie. Twee van de technieken die in het kader van het promotie-onderzoek werden ontwikkeld (Hoofdstuk 2 en 3), werden tevens geschikt gemaakt voor commerciële exploitatie die ter hand genomen is door de firma Sanbio B.V. te Nistelrode.

In november 1979 bezocht hij de afdelingen Farmacologie en Dermatologie van de Universiteit van Newcastle in het kader van een uitwisselingsprogramma van de 'International Union against Cancer'. In september 1981 volgde hij een door de Europese Gemeenschap gesponsorde cursus over 'DNA repair' te Brighton. Tijdens internationale congressen in Halle (DDR), Athene, Helsinki en Stockholm presenteerde hij zijn werk.

Sinds maart 1979 is hij als 'Associate Editor' verbonden aan het tijdschrift 'Ophthalmic Literature'. In 1981 volgde hij een postacademische cursus wetenschapsvoorlichting.

Vanaf 1 februari 1984 is hij werkzaam bij Organon International B.V. te Oss, waar hij o.a. is belast met het beantwoorden van wetenschappelijke vragen van gezondheidsautoriteiten en het samenstellen van de wetenschappelijke produktinformatie voor de marketingorganisatie. Tevens is hij wetenschappelijk adviseur van de Werkgroep voor Cellulaire Differentiatie en Transformatie K.U. Nijmegen.



The design (A) and mounted form (B) of *Epicult*, the new dish for culturing epithelial cells (see Chapter 2).

Nuclesan^{100}, the DNA test kit for measuring DNA in minute tissue samples or small numbers of cultured cells (see Chapter 3).

STELLINGEN

I

De door sommige onderzoekers gevonden zeer grote interindividuele verschillen in de binding van benzo(a)pyreen-metabolieten aan DNA van orgaanculturen worden mede veroorzaakt door de grote variaties in ischaemische schade van het biopsiemateriaal, de aanwezigheid van meerdere celtypen en de korte kweekperiode als gevolg waarvan hormonale, farmacologische en dieetfactoren van de donor nog van invloed kunnen zijn.

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II

De moeilijke reproduceerbaarheid van Kellermann's bevindingen over een relatie tussen AKH-induceerbaarheid in lymfocyten en het bronchuscarcinoom bij de mens, is mede een gevolg van het ontbreken van een maat voor de proliferatie van het gladde endoplasmatisch reticulum gedurende de door mitogenen gestimuleerde lymfoblast-vorming *in vitro*.

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R.E. Kouri, C.E. McKinney, D.J. Slomiany, D.R. Snodgrass, N.P. Wray en T.L. McLemore. Cancer Res. 42: 5030, 1982.

III

Ten onrechte wordt het effect van AKH-inductie op de vorming van dihydrodiol-metabolieten van benzo(a)pyreen *in vivo* en *in vitro* vaak veronachtzaamd.

Dit proefschrift.

IV

Het door Kouri et al. gebruiken van een controlegroep bestaande uit patiënten met niet-maligne longaandoeningen, zoals CARA en tuberculose, lijkt onjuist gezien de relatie tussen deze aandoeningen en het op latere leeftijd ontstaan van een bronchuscarcinoom.

R.E. Kouri, C.E. McKinney, D.J. Slomiany, D.R. Snodgrass, N.P. Wray en T.L. McLemore. Cancer Res. 42: 5030, 1982.

Ten onrechte stellen Maurer en Pfleger dat als gevolg van de door hen gebruikte opwerkprocedure voor de bepaling van metabolieten van anti-depressiva in urine, bij het N-oxide van mianserine een Cope-eliminatie optreedt.

H. Maurer en K. Pfleger. J. Chromatogr. 305: 309, 1984.

G.D. de Jongh, H.M. van den Wildenberg, H. Nieuwenhuyse en F. van der Veen. Drug Metab. Dispos. 9: 48, 1981.

VI

De in 'De Verteller' van H. Mulisch gesuggereerde oorzaak van individuele verschillen in gevoeligheid voor longkanker als gevolg van het roken van sigaretten, is onjuist.

H. Mulisch, De Verteller. p.17-18. De Bezige Bij, 1970.

VII

In semi-aride ecosystemen is de methode van de Frans-Zwitserse school voor vegetatie-analyse doelmatiger dan die van de Angelsaksische school.

M.E. Scholten, M.W.A.C. Hukkelhoven, M.A. Ayyad en M.J.A. Werger. Folia Geobot. Phytotax. Praha, 16: 293, 1981.

VIII

De proton chemical shift van de zure hydroxylgroepen in zeoliet HY gemeten door Freude et al. 1982, is niet in overeenstemming met de recentelijk gepubliceerde waarde.

D. Freude, M. Hunger en H. Pfeifer. Chem. Phys. Letters, 91: 307, 1982.

D. Freude, M. Hunger, H. Pfeifer, G. Scheler, J. Hoffmann en W. Schmitz. Chem. Phys. Letters, 105: 427, 1984.

IX

Waterbeweging in kleigronden kan niet met de bestaande stromingstheorieën worden beschreven.

J. Bouma en J.H.M. Wösten. Soil Sci. Soc. Am. J. 43: 16, 1979.

In de moderne moleculaire biologie wordt vaak ten onrechte veronachtzaamd dat voor biomedisch onderzoek het werken met een voor de *in vivo* situatie zo representatief mogelijk, en daardoor zeer complex modelsysteem, de meest eenvoudige methode vormt voor het op afzienbare termijn verkrijgen van farmacotherapeutisch bruikbare resultaten.

XI

Gezien de discussie die nog gaande is over al dan niet droogleggen van de Markerwaard en de betekenis van het woord 'dijk' (waterkering langs water) in vergelijking met 'dam' (waterkering dwars door water) is de benaming 'Dijk Enkhuizen-Lelystad' voor de nieuwe wegverbinding tussen deze plaatsen op zijn minst voorbarig.

XII

Het beleid van onze centrale overheid ten aanzien van carcinogene stoffen is verbrokkeld over maar liefst vijf verschillende departementen zonder dat er sprake is van een goede coördinatie, noch wat betreft de wetenschappelijke advisering, noch wat betreft de ambtelijke beleidsvoorbereiding.

XIII

De standaardeis tot het uitvoeren van een LD50-test in het kader van acuut toxiciteitsonderzoek bij de registratie van geneesmiddelen, dient te vervallen.

XIV

Het zich de haren uit het hoofd trekken hoeft niet meer uitsluitend door spijtgevoelens te worden ingegeven.

Nijmegen, 21 juni 1984 M. Hukkelhoven